Immune recognition of devil facial tumour disease by the Tasmanian devil

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Declaration

"This thesis contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the thesis, and to the best of my knowledge and belief no material previously published or written by another person except where due acknowledgement is made in the text of the thesis, nor does the thesis contain any material that infringes copyright."

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Publications

Parts of this thesis have contributed to publications. Listed below are these publications, along with author contributions. In all cases the material included in the thesis were performed by the candidate, except where due acknowledgement is made.

Primary supervisor	Signature	Date
Professor Greg Woods		16/12/2016

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AK prepared the table and figures, and refined the manuscript
GW refined the manuscript
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RP, RH & AK carried out field sample collection/ laboratory analysis

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CT & JD performed laboratory analysis and prepared the figures

RM interpreted the histopathology

CT, **RP,** AK, GW drafted the manuscript

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AS, AB, MP adjuvant studies

AP bioinformatics

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Statement of Ethical Conduct

"The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University."

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Abstract

The successful transmission of devil facial tumour disease (DFTD) as an allograft between Tasmanian devils raises many immunological questions about the disease, the devil's immune system and their interplay. Research on DFTD and devil immunology feed into an overarching goal of developing a protective vaccine against DFTD.

The effects of DFTD on haematology and serum biochemistry parameters in the Tasmanian devil have been published. DFTD's effects on the immunological components of the devil's peripheral blood however, continue to be explored. T lymphocyte subsets and immunoglobulins provide useful indicators of immune competence, and have been assessed in the lymphoid organs of healthy devils and those with DFTD. To examine the peripheral blood T lymphocyte subsets of a wild population of Tasmanian devils, a novel method using immunohistochemistry on formalin fixed blood clots was developed. This overcame the limitations of available reagents and the remote field location where samples were collected. An ELISA was developed to measure the relative levels of IgM and IgG in serum from healthy and diseased devils. Devils with DFTD had reduced percentages of CD4+ and CD8+ lymphocytes, and increased IgM and IgG serum levels compared to healthy devils. The effect of age, sex and season on these components was also evaluated in healthy devils. Significant differences between age groups (juveniles and adults) were found for both T lymphocytes and immunoglobulins. Seasonal effects were noted for CD4+ lymphocytes and the CD4:CD8 ratio. There was no detectable sex effect on any of the components.

DFTD is an aggressive cancer with most devils dying within 6 to 12 months of clinical signs first appearing. The long-standing assumption that DFTD always escapes the devil's immune response was addressed by looking for the presence of serum IgG antibodies against DFTD in a population of wild devils. Likewise, tumour biopsies were examined for the presence of tumour infiltrating lymphocytes as an indication of cell mediated immune responses. Approximately 10% of wild devils were capable of mounting an immune response against the disease. This correlated with tumour regression in four out of the six devils with a demonstrated immune response.

The epigenetic down-regulation of the major histocompatibility complex class I molecule (MHC-I) is considered a principle mechanism by which the DFTD cells escape the devil's

immune response. This down-regulation is reversible and DFTD cells incubated with the cytokine interferon gamma (IFN-γ) express surface MHC-I. These cells are expected to be immunogeneic and thus formed the basis for two pilot immunisation trials on a total of six devils, including one adjuvant-only control, and one non-immunised control for live DFTD cell challenge. The immune responses induced by these immunisations were measured. A subsequent challenge with live DFTD cells was given to three of the immunised devils. Tumours developed in two of the devils but subsequent immunotherapy comprising a single subcutaneous injection of live DFTD cells expressing surface MHC-I, resulted in tumour regression in both devils. Serial biopsies of the regressing tumours demonstrated their immune mediated rejection.

The State government's Save the Tasmanian Devil Program's "wild devil recovery" project allowed for the immunisation protocol used in one of the above trials to be carried out on 19 captive held devils prior to their wild release. The relatively large sample size allowed for a more robust assessment of the immune responses measured. There were 15 out of the 19 devils that developed anti-DFTD IgG antibodies in response to the immunisations prior to their release. The effects age and sex had on the responses were also considered. Juvenile (one year old) devils had significantly higher antibody responses than adults, and female devils showed higher antibody responses than males. Inevitable variations in the protocol administered, due to variable trapping success, meant some comparison between the number of immunisations each devil received could also be made. Post release monitoring trips showed serum antibody levels reduced over time in the small number of devils that were retrapped during these trips.

While this thesis was underway, a second transmissible cancer affecting Tasmanian devils was discovered in 2014. This was named DFT2 and bears similar morphological features to DFT1 (the first DFTD). Genetic analyses confirmed that DFT2 arose independently to DFT1. The presence of immune cross-recognition of DFT1 and DFT2 in devils was explored. Both immunised and wild devils with serum IgG antibody responses against DFT1 showed similar responses against DFT2. This suggests that DFT1 and DFT2 have common antigens and a single vaccine could protect against both tumours.

Chapter 1

Literature review

Chapter 1. Literature review

1.1. Introduction

The Tasmanian devil (*Sarcophilus harrisii*) is threatened with extinction in the wild by an aggressive, transmissible and fatal cancer known as devil facial tumour disease (DFTD). The devil is the world's largest extant carnivorous marsupial and unique to the island state of Tasmania. It is listed as endangered by the International Union for the Conservation of Nature as a result of the DFTD epidemic (Hawkins et al., 2008).

The extinction in the wild of the Tasmanian devil would have profound impacts. It is an internationally recognised species with an iconic status and inhabits a unique ecological niche. As devil populations decline this niche is at risk of being filled by feral cats and potentially foxes, the consequences of which would prove disastrous for native species (Hollings et al., 2014, Hawkins et al., 2006).

DFTD is a contagious cancer, passed between devils by biting. A viral aetiology was initially suspected when the transmission pattern became apparent (Ladds et al., 2003), however, it is now clear the tumour cells are the sole aetiological agent (Pearse and Swift, 2006, Murchison et al., 2012). As discussed below, this is a rare event in nature, as the tumour must not only find a way to infect the new host, but also evade the host's immune mechanisms in order to colonise the tissues. This form of tumour transmission is apparent in only one other mammalian species, the domestic dog, which is affected by canine transmissible venereal tumour (CTVT) (Rebbeck et al., 2009). CTVT provides a useful comparison to DFTD. Notably, new transmissible cancers have been recently described in soft-shell clams, suggesting that transmissible cancers might be more common in nature than originally thought (Metzger et al., 2015, Metzger et al., 2016).

Research investigating the devil's immune system and immune responses has shown these to be functional and comparable to other mammalian species. A major limitation of marsupial immunology research is the lack of species specific or cross-reactive reagents such as monoclonal antibodies, leaving many knowledge gaps to be explored. For example, the immune cell populations and serum immunoglobulin levels of devils are largely unknown. These components have been shown to be involved in cancer immunology and therefore have

particular relevance when examining the association between DFTD and the devil's immune system.

While it is useful to draw on the vast body of knowledge acquired from human and rodent immunology studies, the recognition of interspecies differences with respect to immunology is widespread and needs to be considered when assessing the devil's immune system. So too does the effect the environment plays on immune responses, and "wild immunology" as the field that addresses these effects has obvious applications to Tasmanian devil research.

Measures to conserve the Tasmanian devil include the maintenance of a genetically sustainable captive insurance population, the translocation of healthy devils to disease free areas, and research aimed at developing a protective DFTD vaccine. The latter has been underway since 2006. While it is a challenging process, several characteristics of the DFTD cell e.g. its relatively stable nature, make the vaccine approach a worthwhile pursuit. Cancer vaccine development is a rapidly expanding field in medical research and requires the consideration of many possible approaches and options. Most have merit and the vast range of possibilities can be a double-edged sword for DFTD vaccine research particularly given the limitations inherent in working with an endangered species, such as the access to appropriate numbers of devils for suitably powered clinical trials. Detection of immune responses to immunisation trials is another difficulty that arises when working with a species for which there are few appropriate reagents available.

Ever since modelling predicted the likelihood that DFTD would drive devils to extinction (McCallum et al., 2007), human interventions such as those listed above, have been pursued. Perhaps not surprisingly, nature has also responded to the dramatic decline in the devil population with reproductive compensation seeing an increase in precocial breeding, and possibly an increase in female young born to diseased mothers (Jones et al., 2008b, Lachish et al., 2009).

It is encouraging to note that no local devil extinctions have yet occurred, even in the north east of the state where DFTD has been present the longest (Environment, 2016). While there is evidence that devil population bottlenecks have occurred in the past (Bruniche-Olsen et al., 2014), these were due to climate change and hunting, and the species' ability to rebound from the current DFTD driven decline is untested and uncertain. The ecological impacts of the

current population decline are measurable and significant (Fancourt et al., 2015, Hollings et al., 2014, McQuillan, 2009) and therefore human attempts to slow the decline are warranted.

The devil's immune system, DFTD and their interplay provide the opportunity to explore and link the two unique fields of marsupial immunology and transmissible cancers. This research feeds into the overarching goal of DFTD vaccine development, but the process concurrently provides insights into each field. As such, the relevance of this research is likely to extend beyond the devil and DFTD as species extinction, emerging infectious diseases, and diseases as causes of or contributing factors to extinction become more commonplace (Ceballos et al., 2015, Jones et al., 2008a, Macphee and Greenwood, 2013, Heard et al., 2013).

1.2. Origins of DFTD

Cancer is the result of uncontrolled cell division that evades the host's immune surveillance function. Cancer cells typically die with their host, but DFTD has the remarkable feature of being a clonally transmissible cancer (Pearse and Swift, 2006, Murchison et al., 2010), spreading from one individual to the next and outliving its host in the process. CTVT, the only other naturally occurring transmissible tumour of vertebrates, is a sexually transmitted cancer of dogs with a worldwide distribution (Rebbeck et al., 2009) and will be discussed later in this review.

Carcinogens, infectious agents and genetic predisposition are the usual contributors to the cause of cancer. It is unknown what gave rise to the first DFT, but genomic analysis demonstrated this primary tumour appeared in a female devil around 20 years ago (Murchison et al., 2012). DFTD was first observed in 1996 in the far north east of Tasmania (Hawkins et al., 2006) and has since spread to affect the majority of the species' geographic range, and up to 90% of individuals within certain locations (McCallum et al., 2007). It is believed to cause mortality in all affected animals, seemingly within 6 to 12 months of the tumour's appearance (Hawkins et al., 2006). Death results from starvation depending on the size and location of the tumours, or from metastases and subsequent organ failure.

Deep sequencing of the DFTD transcriptome revealed the tumour to be of Schwann cell origin (Murchison et al., 2010). Several genes involved in the myelination of axons are up-regulated in DFTD cells when compared to control tissues. In addition, protein expression was

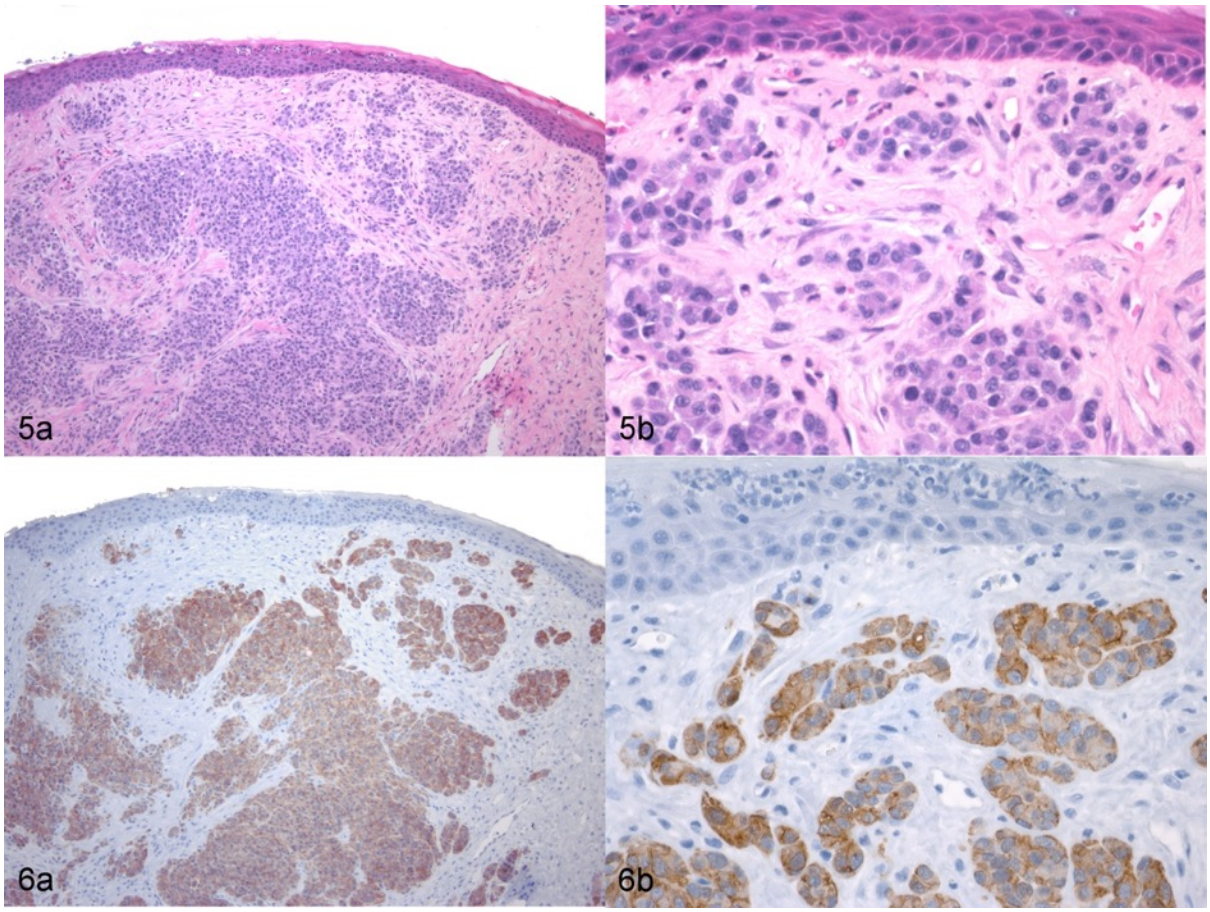
determined by immunohistochemical analyses providing convincing evidence for the Schwann cell origin of DFTD (Tovar et al., 2011).

1.3. Pathology of DFTD

The defining characteristic of DFTD, as the name suggests, is the presence of locally aggressive tumour/s on the facial area. In addition, tumours occur inside the mouth (gingival mucosa, hard palate, lips), and on the head and neck. More than one tumour can be present on a given animal and there is considerable variation in size and external appearance. A DFTD pathology study found the majority of tumours to be well circumscribed, greater than 3 cm, and ulcerated (Loh et al., 2006). The tumours often show epithelial break up, necrosis, exudation and bacterial contamination (Figs 1.1 to 1.3). Metastases are a common finding (65% of cases in Loh's study), and occur primarily in the draining lymph nodes, lungs and kidneys (Fig. 1.4).

DFTD cells have been described as pleomorphic, round to spindle-shaped and with large central nuclei, arranged in nodules or bundles and enclosed by a thin pseudo-capsule (Loh et al., 2006). Variable levels of mitoses, necrosis and poor infiltration of inflammatory cells were also described (Fig. 1.5 a,b). The anaplasia exhibited by the DFTD cells (Loh et al., 2006) is consistent with the highly malignant nature of the tumour (Eberhart and Burger, 2003).

Figures 1.1 – 1.4. Devil facial tumour disease, Tasmanian devil. Figures 1.1 and 1.2. Right upper lip and right cheek, solitary ulcerated mass. Figure 1.3. Right upper gum, multiple ulcerated masses. Figure 1.4. Right and left kidneys, devil facial tumour disease metastases in the cortex of both kidneys.



Figures 1.5 and 1.6. Devil facial tumour disease (DFTD), skin, Tasmanian devil. Figure 1.5. (a) DFTD tumour cells are distributed in the subcutaneous tissues, forming nodules in the dermis and subcutaneous tissue. (b) The neoplastic cells are round to spindle shaped with central nuclei and scant cytoplasm. Haematoxylin and eosin. Figure 1.6. (a) DFTD cells show specific labelling for the Schwann cell marker periaxin. (b) Strong cytoplasmic labelling of DFTD tumour cells with the marker periaxin. Immunohistochemistry for periaxin.

Based on immunohistochemical techniques, DFTD has been classified as a sarcoma, since it is negative for epithelial markers such as cytokeratin, epithelial membrane antigen and von Willebrand factor, and positive for S-100 and vimentin. Further research identified expression of proteins associated with the peripheral nerve system. Importantly, a Schwann cell marker, periaxin, was found to be highly expressed in 100% of primary DFTD tumours, DFTD metastases, cultured DFTD cells and mouse DFTD xenografts (Fig. 1.6 a,b) (Tovar et al., 2011). Corroborating this finding, gene expression analyses of DFTs identified an overexpression of genes related to the myelination pathway of peripheral nerve tissue, specifically Schwann cells (Murchison et al., 2010).

An independent review of chemical residues found in healthy devils and those affected with DFTD was commissioned by the Save the Tasmanian Devil Program (STDP) (Ross, 2008). The chemicals selected for investigation included heavy metals, herbicides and pesticides. Residues of dioxins, dibenzofurans, polychlorinated biphenyls (PCBs), brominated diphenyl ethers, arsenic, cadmium and lead were detected in the fat and/or liver of most animals but at levels similar to those found in other species at the top of the food chain, including humans. There were no significant differences in residue levels between the healthy and diseased devils and the author concluded there was no apparent link between these chemicals and DFTD.

1.4. Allograft theory of DFTD transmission

Transmissible cancers are an extremely rare occurrence in nature but evidence for the clonality of DFTD is conclusive, both from karyotypic and genetic perspectives. Pioneering research on DFT chromosomes demonstrated that the karyotypic rearrangement of 11 DFTs was complex and identical for each of the tumours examined. The karyotype of DFTD tumour cells showed several abnormalities, including the loss of both chromosomes 2 and both sex chromosomes, and the addition of four new unidentified markers. Since it is theoretically impossible that each DFT acquired the same complex rearrangement by chance it was concluded that DFTs are clones derived from the same original tumour and the allograft theory of disease transmission was put forward. In addition, the authors identified one host with a pericentric inversion of one of the chromosomes 5, but its DFT contained no such inversion, demonstrating that this DFT could not have arisen from its host (Pearse and Swift, 2006). This clonal hypothesis was supported by chromosome painting which further characterised the marker chromosomes showing them to be derived from chromosomes 1, 5 and X (Deakin et al., 2012). These authors

noted that chromosomes 1 were mislabelled as chromosomes 2 in the original research. Chromosome painting also identified significant regions of homology between normal and DFT chromosomes.

The genetic evidence supporting DFT's clonal origin has been acquired from microsatellite and MHC genotyping as well as whole genome analysis. Matched tumour and host samples from 15 devils, and blood samples from 11 non-diseased individuals were genotyped at four polymorphic microsatellite loci, and MHC class I and II loci. While 90% of sampled devils had unique genotypes, all the tumours were identical at multiple microsatellite and MHC loci, supporting the tumour's clonal nature (Siddle et al., 2007). A larger study of 25 matched tumour and host samples, and 10 samples from non-diseased devils were acquired from 16 locations across Tasmania. Fourteen microsatellite loci were genotyped and all tumours shared a comparable genotype across all loci, independent of location, sex or age of the devil (Murchison et al., 2010). Both studies found that the tumour genotype was distinct from that of the host devils. Thus it would be impossible for DFTs to have arisen from the host's own tissues and consequently supporting the tumour allograft theory.

Finally, whole genome analysis (Miller et al., 2011, Murchison et al., 2012) further substantiated the tumour's clonal origin and allograft theory of transmission by demonstrating that DFTs share structural variants and copy number changes distinct from their hosts.

It is relevant to note here the contribution of telomere length and telomerase activity to the continued proliferation of DFT cells. Ujvari et al suggested the cells monitor and regulate the length of individual telomeres to favour their genomic stability and possibly increased proliferation (Ujvari et al., 2012).

1.5. Evidence for direct tumour cell transmission

The successful experimental induction of DFTD in naïve devils by the transfer of cultured tumour cells and/or cells from primary DFTs took place several years ago, although this has not been formally described (Pyecroft et al., 2007). More recently, two captive devils were challenged with live DFTD cells following an immunisation trial. Eventually both devils developed palpable tumours from 25,000 live DFTD cells. These experiments supported the theory that DFTD cells are successfully transferred from an infected devil to another (Kreiss

et al., 2015). The identification of viable tumour cells on the canine teeth of DFTD infected devils provided evidence for the natural mechanism of DFTD transmission (Obendorf, 2008).

The closest relatives of the Tasmanian devil are other members of the Dasyurid family, including the spotted-tailed quoll (Dasyurus maculatus) and the eastern quoll (Dasyurus viverrinus), both of which are present in Tasmania. There is no evidence of DFTD naturally occurring in these species although transmission trials have not been performed. DFTD transmission trials were carried out on mice, but only severely immunocompromised mouse strains developed tumours after implantation (Pinfold et al., 2014).

These independent lines of evidence convincingly characterise DFTD as a transmissible tumour that acts as an allograft and evades the host immune system. There are three possible explanations for how a tumour allograft could establish in the devil population:
1. devils have a poor immune response or are immunosuppressed;
2. devils are matched at the Major Histocompatibility Complex, or
3. the tumour cells evade the host's immune system.
Each of these theories has been explored and are summarised below.

1.6. The Tasmanian devil's immune response

The early consensus that marsupials had a primitive immune system has been overturned with current research showing their immune response to be closely akin to that of eutherian mammals (Belov et al., 2013). The overall similarity of the mammalian immune systems with respect to development, components and complexity is noteworthy given that divergence of marsupials from their eutherian counterparts occurred 148 million years ago (Bininda-Emonds et al., 2007).

1.6.1. Innate immune response of the Tasmanian devil

Tasmanian devils are carnivores, specialised scavengers and opportunistic predators. Their diet and biting behaviour expose them to a wide range and high level of bacteria and parasites, yet there is little evidence that wild devils succumb to disease of significance from such pathogens (Obendorf et al., 1990). It is logical to assume that devils have fully functional innate

phagocytic and humoral immune responses since these provide primary protection against bacterial and parasitic pathogens.

Unsurprisingly, the devil's neutrophils have been shown to be highly efficient at phagocytosis. Using functional assays such as the production of oxidizing compounds to kill bacteria and the demonstration of phagocytic uptake and killing of E. coli, this aspect of their innate immune response was shown to be proficient (Kreiss et al., 2008).

1.6.2. Humoral immune response of the Tasmanian devil

B cells producing immunoglobulin (Ig) M and IgG are present in devil lymphoid tissue (Howson et al., 2014). Devils rapidly responded to immunisation of horse red blood cells, with a single injection resulting in measurable IgG antibody titres after one week (Kreiss, 2009). The relatively high titres were maintained throughout the eight month period of testing. A booster given six months after the first immunisation resulted in strong secondary responses. These results provided evidence that the devil has competent humoral immune responses and memory. Demonstrating the devil's ability to mount an antibody response against xenogeneic tumour cells, Tasmanian devils immunised with the K562 human leukaemia cell line developed high antibody titres following a second immunisation (Brown et al., 2011). The usefulness of serum IgM and IgG analysis with respect to devil immunology and DFTD research, and the presence of anti-DFTD IgG antibodies are discussed later in this review.

1.6.3. Cell mediated immune response of the Tasmanian devil

Although Tasmanian devils resist most bacterial and parasitic insults, they and other dasyurids have a higher incidence of tumours compared to other marsupial families (Attwood and Woolley, 1973, Griner, 1979, Canfield et al., 1990). Cell-mediated immunity plays a crucial role in both tumour and allograft rejection and in light of the devil's susceptibility to tumours, and the allograft nature of DFTD, a thorough understanding of the devil's cell mediated immune response is required. Detailed investigation and clarification of immune cell function is significantly hampered by the lack of species-specific reagents (Belov et al., 2013). For example, antibodies against devil CD4 and CD8 T cells have only recently been developed and only for use in immunohistochemical preparations (Howson et al., 2014). The applications for

peripheral blood analysis of CD4+ and CD8+ lymphocytes in other species, and how these would contribute to devil immunology and DFTD research are addressed later in this review.

T cell proliferation assays revealed the devils to have a relatively robust mitogen-induced response confirming T cell immune capacity (Kreiss et al., 2008, Stewart et al., 2008). Notably, devils affected with DFTD had similar mitogen-induced responses as their disease-free counterparts suggesting that immune suppression, at least at the level of lymphocyte proliferation, does not explain susceptibility to DFTD.

In addition to demonstrating antibody responses to K562 cells, Brown et al also provided evidence of strong cytotoxic responses against the K562 cells in the presence of immune serum (Brown et al., 2011). Antibody dependent cell mediated cytotoxicity (ADCC) carried out by natural killer (NK) cells provides an explanation for this, and implies a possible route for inducing an anti-DFTD response. It is unclear whether NK cells can be activated against DFTD cells in the presence of anti-DFTD antibodies.

While there are gaps in the knowledge, all research to date suggests the devils have a fully functional immune system comparable to that of other mammals, and a limited immune response does not explain the successful transmission of DFTD.

1.7. Genetic diversity and the Major Histocompatibility Complex (MHC)

Low genetic diversity of Tasmanian devils is expected given they are an island species (Frankham, 1997), and devils have been shown to exhibit low heterozygosity and allelic diversity at microsatellite loci (Jones et al., 2004). However, these results give information on population history e.g. relatedness and previous population bottlenecks, whereas analysis of MHC diversity better represents a population's "fitness" and ability to counter disease challenges.

The MHC is a cluster of genes occurring in all vertebrates and is the most polymorphic portion of the mammalian genome. These genes were first associated with foreign tissue (allograft) transplantation, but are now known to be essential in the immune recognition of pathogens and tumour cells. In addition, MHC molecules provoke vigorous T cell responses against incompatible cells and regulate the immunological mechanisms of tissue graft rejection (Li and

Raghavan, 2010). The diversity of MHC genes (i.e. polymorphism) provides the foundation for specific immune responses against infectious agents such as bacteria and viruses.

A diversity of MHC molecules allows presentation of different antigen fragments from the infectious agents to a diverse range of T cells. This produces a stronger immune response than that when only a few antigen fragments are presented. Thus, MHC diversity contributes to disease resistance within a species. However, it was less the infectious nature of DFTD and more so its similarity to an allograft and consequent failure to be rejected by the Tasmanian devil's immune system that put the spotlight on the devil's MHC.

Low diversity of MHC class I (MHC-I) in Tasmanian devils has been verified by sequence analysis (Siddle et al., 2007). An exploration of the MHC diversity in historical and ancient devil samples showed very similar levels to the modern devil population suggesting that low MHC diversity has been a feature of the species for the last 10,000 years (Morris et al., 2013). This research supported the intuitive explanation that low genetic diversity in devils may be a factor that makes devils more susceptible to allograft acceptance. This was, however, countered by subsequent skin graft transplant experiments (Kreiss et al., 2011). Cheetahs are another example of a wild species recognised as having extremely low genetic diversity, and allogeneic skin grafts between cheetahs were performed to test MHC variation (O'Brien et al., 1985, Sanjayan and Crooks, 1996). Of the 14 cheetahs, only three showed signs of rejection, which took at least 40 days. Monomorphism at the MHC complex was suggested as the probable cause. In contrast, allogeneic skin graft experiments performed on Tasmanian devils (Kreiss et al., 2011) found that all seven recipient devils rejected the grafts within 14 days. The immunologic mechanism of the rejection was confirmed by the characteristic infiltration of CD3+ lymphocytes. This suggests competent T-cell activity against the allografts, implying the immune system of individual devils recognise foreign MHC and should have the potential to mount a response against DFTD.

This demonstration of a functional immune system and sufficient genetic diversity to reject allografts (other than DFT) suggests that intrinsic tumour properties are responsible for escaping the devil's immune response.

1.8. Immune escape mechanisms of DFTD

1.8.1. Overview of tumour immune escape mechanisms

Immune escape mechanisms have been recognised in a wide variety of tumours including melanoma, mammary carcinoma and various adenocarcinomas (Seliger et al., 2001, Kim et al., 2007). These include down-regulation or defective expression of MHC-I, down-regulation or defective mechanisms of antigen processing, and secretion of immunosuppressive cytokines. Inhibition of antigen presenting cells, in particular dendritic cells, is recognised as a significant immune escape mechanism of tumours in people and mice (Tourkova et al., 2009).

1.8.2. Down-regulation of MHC-I

Since 2007 DFTD cells have been known to possess MHC- I transcripts (Siddle et al., 2007). The presumption that MHC-I was expressed on the DFTD cell surface supported the hypothesis that the devils, with their reduced MHC diversity, did not identify the tumour cells' foreign MHC, thus allowing successful establishment and proliferation of the tumour. It has since been demonstrated however that DFTD cells down-regulate expression of their MHC genes and this represents a key mechanism by which the tumour evades immune system detection (Siddle et al., 2013). Of great significance is that expression of MHC-I molecules can be restored on DFTD cells both *in vitro* and *in vivo*. The former has been demonstrated by treating cells with the cytokine interferon gamma (IFN-γ). The *in vivo* expression of MHC-I has been demonstrated, albeit rarely, on DFTD biopsies which have positive staining with immunohistochemistry for beta 2 microglobulin (a component of MHC-I). This finding has implications for vaccine development and will be discussed further in this review.

1.8.3. Immunosuppressive cytokines and inhibition of antigen presenting cells

Secretion of immunosuppressive cytokines by tumours of Schwann cell origin has been demonstrated as a mechanism for escaping immune detection (Watanabe et al., 2001). The tumour-promoting role of transforming growth factor beta (TGF-β) in the microenvironment of human cancers has been reviewed (Jakowlew, 2006, Moutsopoulos et al., 2008, Kaminska et al., 2005) and its significant role in the progression and regression of experimentally induced CTVT has been explored (Hsiao et al., 2008). The anti-inflammatory cytokine interleukin 10 (IL10) has a complex and vast array of functions but its significance with respect to immune

escape lies in its ability to suppress both T cell proliferation and the production of inflammatory cytokines such as interleukin 1 (IL1) and tissue necrosis factor (TNF), and to impede the roles of antigen presenting cells (Sato et al., 2011).

Identification of IL10 and TGF-β transcripts in the DFTD transcriptome suggested that synthesis of cytokines by DFTD cells could allow evasion of the devil's immune response and enhance tumour growth. This was supported by unpublished immunohistochemistry results for DFTD biopsies that detected IL10 and TGF-β in the DFTD tumour cells (Howson, 2011). Quantitative PCR results did not show up-regulation of these cytokines nor of vascular endothelial growth factor A (VEGF-A) or interleukin 6 when compared to normal devil tissue (Morris and Belov, 2013). However, since cytokine concentrations as low as 0.1 ng/ml are active, and since tumor biopsies include non-neoplastic cells, a more precise method to assess protein expression by DFTD cells would be valuable as a complementary method to quantify these cytokines (Siddle and Kaufman, 2015, Woods et al., 2015).

The presence of immature, but absence of mature dendritic cells within the DFT stroma has been noted and it was hypothesised the tumour cells, potentially via TGF-β, are actively preventing dendritic cell maturation (Howson et al., 2014). TGF-β has also been associated with the development of tolerogenic dendritic cells. Lastly, TGF-β inhibits the function of NK cells, which would typically recognise cells not expressing MHC-I as abnormal and eliminate them.

In summary, evidence demonstrates that devils have an effective immune response and adequate MHC diversity to recognise and reject allografts. This suggests that the lack of immune recognition of DFTD is due to the tumor cells' immune escape mechanisms. Down-regulation of MHC by DFTD cells is possibly the most important of these mechanisms allowing for DFTD transmission between devils.

1.9. Different strains and ploidy of DFTD

Four strains of DFTD have been identified on the basis of their different karyotypes (Pearse et al., 2012). These authors suggest that the variants are likely to have different biological characteristics with respect to factors such as transmission and virulence, although there are few corroborating data for this. Based on chromosome painting and gene mapping, it has been

suggested that the minimal cytogenetic differences among tumor strains may not have clinical implications (Deakin et al., 2012). An increase in tetraploidy was observed in DFTs in devils from the Forestier Peninsula where disease suppression by selective culling was trialed (Ujvari et al., 2014). Since polyploid cells are often larger and may be slower to divide (Otto, 2007) this could have consequences for tumor growth. Because devils were removed from that site, long-term effects of tetraploidy in that population could not be assessed. The influence of ploidy was observed in a longitudinal study in northwest Tasmania where a high initial prevalence of tetraploid tumors in the study site was associated with low DFTD infection rates and limited host population effects. When the diploid DFTD variant reached the site, it replaced the tetraploid variant, causing disease prevalence and population effects to rapidly increase (Hamede et al., 2015).

1.10. Transmissible cancers, and a comparison between CTVT and DFTD

Prior to 2015, the only naturally occurring transmissible cancers known to exist were CTVT in dogs, and DFTD in Tasmanian devils. However, in 2015-16, six more transmissible cancers were reported. These were five cases of disseminated neoplasia in different species of molluscs (Metzger et al., 2015, Metzger et al., 2016); and Devil Facial Tumour 2 (DFT2), a second transmissible facial cancer affecting Tasmanian devils (Pye et al., 2016). DFT2 is grossly indistinguishable from the original DFTD, but the two tumours have distinct histopathological features and karyotypes, and they differ from each other and their host devils at microsatellite loci, structural variants and at MHC loci. The karyotype of DFT2 contains an X and a Y chromosome indicative of a male origin which contrasts to the female origin of DFT1 (Pye et al., 2016, Murchison et al., 2012).

CTVT is an estimated 11,000 years old (Murchison et al., 2014) and has been the subject of extensive research since its transmissible nature was first noted in 1876. CTVT provides a useful comparison with DFTD given some striking similarities and pronounced differences. The following is a brief summary of three reviews comparing CTVT and DFTD (Murchison, 2008, Siddle and Kaufman, 2012, Belov, 2012).

DFTD's malignant and fatal nature is the most pronounced difference between it and CTVT. Typical cases of CTVT rarely metastasise or cause fatality in immunocompetent hosts. Interestingly the tumours share the same immune escape mechanism of MHC-I down-

regulation as they establish themselves in the host. This "progressive phase" of CTVT whereby only 3% of tumour cells express MHC-I is followed by the stationary or regressive phase, characterised by cessation of tumour growth. This phase is associated with increased expression of cell surface MHC-I, lymphocyte infiltration and increased levels of the host-derived IFN-γ (Hsiao et al., 2008). The fact that MHC-I expression can be restored on both CTVT and DFTD cells demonstrates lack of expression is due to regulatory mechanisms rather than structural defects and has obvious implications for tumour recognition by the host immune system. Restoration of MHC-I expression occurs during the course of CTVT infection, suggesting this tumour has reached an equilibrium with its host, allowing the survival of both. This balance does not exist between DFTD and the devil. DFTD is currently driving its host toward extinction and by default itself. If time allows, DFTD may evolve to a less aggressive disease with up-regulation of MHC-I (reflecting the course of CTVT) providing one possible outcome of evolutionary development.

Serum IgG antibody against CTVT cells has been demonstrated in CTVT-affected dogs, suggesting a humoral immune response to the disease (Cohen, 1980, Cohen, 1972). A humoral immune response in the form of IgG antibodies against DFTD cells was also evident in devils undergoing a DFTD immunisation trial (Kreiss et al., 2015). While cell-mediated immunity is primarily responsible for anti-tumor activity, IgG antibody production against DFTD is a significant finding in a disease characterised by its successful immune escape mechanisms.

1.11. T lymphocytes and immunoglobulins: indicators of immune function; the effect of cancer

1.11.1. T lymphocytes

The CD4+ T helper cells and CD8+ cytotoxic T lymphocytes have critical roles in cell mediated and anti-tumour immune responses, but they also act as indicators of immune function. The ratio of CD4:CD8 cells in the peripheral blood is used frequently in human medicine as an indicator of immune competence. This applies particularly to HIV infection but a reduced ratio is also associated with a variety of conditions including viral, parasitic and bacterial infections, trauma and malnutrition (Walker and Warnatz, 2006).

Lymphopenia is a common finding in advanced cancer patients and all lymphocyte subsets are affected, in particular CD4+ lymphocytes (Whiteside, 2006, Kuss et al., 2004). Not only are there fewer T lymphocytes in patients with malignancies, but they show reduced responses to antigens and mitogens (Whiteside, 2006). This suggests cancer has an adverse effect on immune competence, and potentially increases susceptibility to infection (Kuss et al., 2004).

The T lymphocyte subset populations are also affected by season, age and sex in humans, and this has been explored in some other species (Heaton et al., 2002a, Heaton et al., 2002b, Martin et al., 2008, Fares, 2013). A seasonal effect on total lymphocyte counts in healthy adult male Tasmanian devils has been recorded (Peck et al., 2015) although the same study found no significant differences between age groups or sex on lymphocyte or total white cell counts.

Analysis of the peripheral blood T cell subpopulations has been used in human medicine to understand and predict organ transplant (allograft) rejection (Miqueu et al., 2010, Kreijveld et al., 2008, Cravedi and Mannon, 2009) suggesting this would also provide insights into DFTD's behavior as an allograft. Patients experiencing organ rejection crises were shown to have significantly higher CD4:CD8 ratios than patients with stable graft function (Kiparski et al., 1990). A study to determine the appropriateness of reducing immunosuppressive therapy in patients following renal transplant found that an increased CD4:T regulatory cell (Treg) ratio prior to cessation of medication was a strong predictor of graft rejection following medication withdrawal (Kreijveld et al., 2008). An alteration in naïve, effector and memory T cells over time was also observed in graft rejectors.

CD4+ and CD8+ T lymphocytes have been identified in the lymphoid organs of healthy devils and those with DFTD (Howson et al., 2014). Due to a lack of appropriate reagents, T cell subset analysis of the peripheral blood of devils and how they are affected by DFTD has not been explored.

1.11.2. Serum immunoglobulins

The serum levels of plasma proteins such as IgM and IgG are altered in response to certain conditions including infection, trauma and neoplasia (O'Connell et al., 2005). The effect of DFTD on total protein, albumin and globulin levels in the serum of devils has been assessed (Peck et al., 2015, Peck et al., 2016) and examination of DFTD's association with serum IgM

and IgG levels has been approached at the RNA level (Ujvari et al., 2016). Exploration of the globulin fractions in serum of devils with DFTD has not been carried out, and nor has DFTD's effect on serum IgM and IgG at the protein level.

While it was originally thought that immunoglobulins were only produced by B lymphocytes, the production and secretion of IgG by various epithelial cancerous cells (lung, liver, prostate, breast) has since been demonstrated (Niu et al., 2012, Chen and Gu, 2007, Qiu et al., 2003). These cancerous immunoglobulins (cIgG's) are believed to have a dual function, promoting growth and proliferation of the cells, as well as providing protection against anti-cancer components of host serum (Lee, 2014). Past experiments have inadvertently found no evidence that DFTD cells secrete IgG. For example, the no serum controls for an IgG response against DFTD cells in both flow cytometry and ELISA were negative (Kreiss et al., 2015). Likewise, there was no evidence for the presence of IgG when DFTD sections were stained via immunohistochemistry (Howson et al., 2014).

1.12. Anti-cancer antibodies in serum and their prognostic value

A large number of tumour associated antigens (TAA's) have been explored with respect to the humoral immune responses they induce and the associated prognostic value of the responses (Whiteside, 2006, Reuschenbach et al., 2009). Cell mediated (CMI) rather than humoral immunity tends to play the primary role in tumour immunity, particularly with respect to cytotoxic lymphocyte activity. However, demonstration of an antibody class switched humoral immune response against cellular immunogens is indicative of a CD4 T cell response. This typically occurs via presentation of peptides on the MHC class II molecules of antigen presenting cells (APCs) to T helper cells which then provide "help" to B cells and the subsequent proliferation of B cells and antibody production.

Some antibodies against TAA's seem to be markers of exposure or immunopathology rather than showing any functional relevance or protective role. Antibodies against p53 (tumour suppressor protein) and MUC1 (an epithelial mucin overexpressed in the majority of adenocarcinomas) have been explored for their prognostic value. Mutations of p53 are found in more than half of all cancers, and poor survival of patients with p53 antibodies has been demonstrated in many studies (Reuschenbach et al., 2009). In contrast, antibodies against MUC1 are associated with improved survival in cancer patients. It has been suggested that

cancer vaccines targeting the MUC1 antigens might benefit from addressing CD4 and B cell responses as well as cytotoxic cellular immune responses (Reuschenbach et al., 2009).

Evidence of antibody production correlating with anti-tumour activity has been observed in human cases of breast and pancreatic cancers both of which express MUC1 antigens, as well as CTVT (Blixt et al., 2011, Hamanaka et al., 2003, Cohen, 1972). On the other hand, antibody responses in melanoma patients were associated with a poor prognosis (Zornig et al., 2014).

The prognostic value of anti-DFTD antibodies is currently unknown. However, the relative ease of identifying antibody compared with cytotoxic responses applies particularly to wild animals for which sample collection and processing can be challenging, and for which there are few species-specific reagents available. Although serum antibodies against DFTD can be induced in immunised devils (Kreiss et al., 2015) there has been no convincing evidence of an antibody response against DFTD in wild devils. Since there are few examples of disease being the primary cause of extinction of a species, it is not unexpected that some wild devils will survive a DFTD challenge presumably by way of a detectable immune response.

1.13. Comparative immunology and wild immunology

Comparative immunology is a discipline recognised since the 1960's and is defined as the analysis of shared and diverging aspects of immunology among species (Wang et al., 2009). Evolutionarily distant species share several immunological features some of which are quite subtle, for example the conservation of TGF-β, vascular endothelial growth factor (VEGF) and various chemokines e.g. interleukin (IL) 8 in invertebrates and vertebrates. Growth factor and cytokine function can be shared across vertebrates as illustrated by human TGF-β's effect on the function of fish macrophages (Conrad et al., 2007).

In contrast, differences can occur between quite closely related species. Human and mouse immune systems are the most widely studied but do not always reflect those of other species. For example, both pigs and ruminants differ notably to humans in their T lymphocyte populations. These species have higher numbers of γδ T cells in the peripheral blood (up to 25% in adult cattle, 60% in calves) compared to humans and mice (1 - 5%). In humans, the function of these γδ T cells is cytokine production, antigen presentation and immune regulation

whereas in ruminants they have a predominantly regulatory role (Guzman et al., 2014). Regarding CD4+ and CD8+ T lymphocytes, healthy pigs and ruminants have a significant peripheral blood population of double negative cells, and pigs also have a double positive cell population. Pigs have a higher percentage of CD8+ than CD4+ lymphocytes in their peripheral blood and lymphoid organs i.e. the inverse proportion of humans, mice and domestic species. They also have an inverted lymph node structure whereby the typical medullary tissue is external to the follicle-containing "paracortex" (Binns, 1982).

The purpose for such variations is not clear, but presumably they provide novel mechanisms for antigen recognition and roles for regulatory function. While it is easy to find striking examples of conservation across species with respect to the immune system, differences are also apparent. These differences highlight the imprudence of automatically translating immunological findings from one species directly to another.

Marsupials and eutherian mammals diverged nearly 150 million years ago so while variations are expected, research to date has found remarkable immunological similarities between the two groups. Many immune system components in marsupials are comparable to other mammalian species including lymph node architecture, cytokine production, and the role of MHC (Belov et al., 2013). The first marsupial species to have the presence of CD4 and CD8 confirmed in their tissues were the opossum (Monodelphis domestica) and the tammar wallaby (Macropus eugenii). Signature features of both eutherian molecules were maintained in the marsupials (Duncan et al., 2007, Duncan et al., 2009).

Differences between marsupials and eutherians include the lack of immunoglobulin D (Miller, 2010), a single IgG allotype (Sun et al., 2013), and a T cell receptor (TCRμ) unique to marsupials (Parra et al., 2007). Little is known about the peripheral T cell subset populations of marsupials due to a lack of reagents. The recent development of an anti-koala monoclonal antibody binding fresh CD4+ T cells has allowed the characterization of this population of cells in healthy and diseased koalas (Mangar et al., 2016). The ability to do the same for devils would provide greater insight to their immune system. Not only would it allow for comparison with other species, but it would inform how the devil's T cell population, critical for cell mediated immunity and anti-tumour responses, is affected by DFTD.

Comparative immunology has some crossover with wild immunology which is a relatively recent discipline that has emerged from the increasing recognition that immunity is affected by exposure to a wide range of pathogens (Jolles et al., 2015, Pedersen and Babayan, 2011, Babayan et al., 2011). Wild immunology has been proposed as the "missing link between laboratory based immunology and human, wildlife and domesticated animal health" (Pedersen and Babayan, 2011). While experiments on inbred strains of laboratory animals have made great contributions to immunology research, the highly controlled laboratory environment ignores the effects that genetic and environmental diversity play. These elements of diversity are central to wild immunology. The inherent limitations in studying wild animals include a lack of specific reagents and annotated genomes, along with the statistical constraints associated with individual and population variation. Much of this applies to Tasmanian devil immunology research, although the annotated genome is available (Murchison et al., 2012).

Despite the limitations of wild immunology, successful studies have been carried out. For example, the wild population of Soay sheep was shown to demonstrate superior antibody responsiveness in association with reduced reproduction but increased survival in adult females i.e. immune and reproductive system tradeoffs (Graham et al., 2010). Antibody responses in wild compared to captive hyenas have highlighted the importance of environmental modulators of immunity (Flies et al., 2015).

As alluded to, marsupial immunology research straddles both fields of comparative and wild immunology. DFTD has necessitated considerable advances in the understanding of the devils' immune system and as such devil immunology has made notable contributions to these fields. The same can be said for koala immunology. Koalas share the status of iconic Australian marsupial with the devils, and are faced with disease threats from chlamydia and koala retrovirus (KoRV) just as devils are threatened by DFTD. Likewise, a chlamydia vaccine for koalas is being pursued just as vaccine development is one of the adopted approaches to address DFTD. The parallels between koala and devil immunology research are marked, particularly with respect to exploring immune system responses of marsupial species for which few specific reagents are available. Together they exemplify the challenging and innovative disciplines of comparative and particularly wild immunology.

1.14. Epidemiology of DFTD and compensatory responses of devils to population decline

DFTD was first observed in 1996 at Mount William National Park in the state's far northeast, and has since spread to cover the majority of the state. Figure 1.7 shows its current distribution. Most pathogenic diseases are dependent on a critical density of their host population for transmission so when a population falls below a certain density, the causal pathogen dies out. However, DFTD prevalence is maintained in devil populations that have suffered significant (up to 90%) declines, which strongly suggests that DFTD follows a frequency dependent pattern of transmission (McCallum et al., 2009). This transmission pattern is typical of sexually transmitted diseases (STDs) and field studies indicate that bite wounds peak during the mating season giving DFTD characteristics of an STD (Hamede et al., 2008).

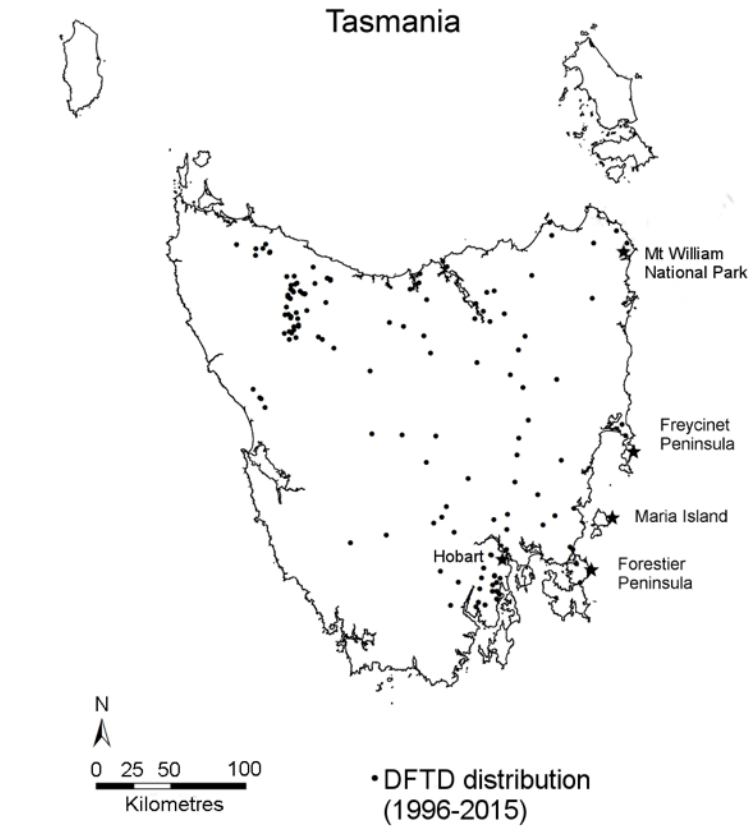


Figure 1.7. Map of Tasmania showing the distribution of devil facial tumour disease (DFTD) from 1996 to 2015. Each dot represents 1 case of DFTD confirmed by histology; stars represent locations mentioned in the text.

There is a high DFTD prevalence (at least 50%) amongst adult devils in populations where disease is well established (McCallum et al., 2009) and it is rare to find devils over three years of age in these areas despite a natural life expectancy of up to six years in the wild. There is no consistent evidence to show a difference between the sexes in DFTD prevalence (McCallum et al., 2009).

A rise in precocial breeding by female devils in diseased populations, explained by increased food availability and growth rates resulting in earlier sexual maturity, demonstrates reproductive compensation for population decline (Lachish et al., 2009). These authors also noted that diseased females showed a propensity for producing female biased litters when compared to their healthy sisters suggesting that sex allocation bias is a result of DFTD infection.

DFTD driven population decline may result in selection for more submissive individuals since it is the more aggressive devils that are likely to acquire infection (Hamede et al., 2013). However, this may be offset by the likelihood that more aggressive males will breed successfully. One study that explored particular regions of the devil genome in three geographically distinct populations found concordant signatures of genetic selection in all three populations after DFTD had entered the populations (Epstein et al., 2016). This hints at gene selection in response to DFTD and further research will determine if there is any association between those genes and increased devil survival or indeed DFTD resistance.

1.15. Management options available to prevent species extinction

A range of management options has been considered to save the Tasmanian devil from extinction which are summarised in Table 1.1 along with their advantages and disadvantages. Some options, such as captive breeding and translocation have already been successfully implemented, whereas others have either proved unsuccessful e.g. disease suppression, or are being currently researched e.g. vaccine development.

This review has already alluded to DFTD evolution, and certainly the development of a less aggressive disease would favour survival of the host and by consequence the disease. It is possible that a more benign form of DFTD will evolve given enough time and devil hosts, and it is conceivable that implementation of certain management options might interfere with the

evolutionary process. However, given the rapid and devastating effects of DFTD to date with no real evidence for these abating, it would be irresponsible to neglect intervention and let the disease take its course knowing the implications of devil extinction.

Table 1.1. Proposed management tools to save the Tasmanian devil from extinction

Management tool	Reasoning	Advantages	Disadvantages
Captive breeding	Captive breeding provides an insurance population in case a species becomes extinct in the wild. One of the first initiatives to save the Tasmanian devil from extinction was the establishment of a captive insurance population, which currently holds approximately 500 individuals. Ideally 95% of the genetic diversity present in the founder population is represented within these captive devils. Different management breeding options utilised include: Intensive breeding; devils are kept in isolation or small groups and mate selection is strictly controlled during the breeding season Extensive breeding; devils are kept in groups in large double-fenced areas. Breeding is loosely controlled, mate choice is largely defined by the animal	Eliminate risk of DFTD with appropriate quarantine regulations Target breeding to preserve genetic diversity Availability of individuals for research projects to further the understanding of devil and DFTD biology	Expensive Devils might lose 'wild' behaviour Decreasing fecundity with each generation Genetically important animals might not breed Post-breeding animals require ongoing care
Vaccine development	An effective vaccine against DFTD might allow the repopulation of DFTD-affected areas with vaccinated devils from the current captive insurance population. It is conceivable that disease-free wild devils could be vaccinated in significant numbers given that many populations have proven amenable to trapping and handling. One encouraging aspect regarding the likelihood of vaccine development is the high conservation of tumour morphology and genotype implying that tumour antigens are conserved, thus giving the immune system a stable target. (Woods et al., 2015)	Opportunity to prevent disease Possibility to induce 'herd immunity' Complements other management strategies The process of vaccine research improves the understanding of devil immunology and DFTD. This knowledge could be applicable to other marsupials and other transmissible cancers	Expensive to produce and to deliver Need to trap the animals for injection Current research suggests the need for several boosters It might change disease dynamics and DFTD evolution
Population reinforcement	This term refers to the reintroduction of a species to its indigenous habitat with the aim of enhancing the local population. Several areas of Tasmania have had drastic declines of the devil population following DFTD appearance and these areas would be suitable for reinforcement.	To augment the local population and thus re-establish the ecosystem balance that existed prior to DFTD's arrival Improve genetic diversity	Reintroduced healthy devils will be at risk of contracting DFTD Devils might disperse elsewhere.

Management tool	Reasoning	Advantages	Disadvantages
Translocation	The first translocation of Tasmanian devils occurred at the end of 2012, to Maria Island. Other islands offshore of Tasmania are being considered.	Islands' natural boundaries prevent the incursion of diseased animals Devils can retain 'wild behaviour' Once the population is established, reduced management compared to other populations.	Impact on other species and the ecosystem balance Need to monitor for inbreeding, overpopulation and welfare.
Disease suppression through culling	A disease suppression trial took place in a semi-isolated peninsula in the southeast of Tasmania between 2004 and 2010. DFTD's prevalence remained the same as other unmanaged sites. This management tool is ineffective because of the long incubation period, frequency-dependent nature of DFTD, no current pre-clinical test and the failure to capture trap-shy animals. (Beeton and McCallum, 2011)	Possibility to keep a functional population in its original area Animals can be euthanised before the onset of severe clinical illness.	Unfeasible unless a high proportion of the population is trapped(Beeton and McCallum, 2011) It does not allow for natural resistance to develop Possible change in tumour biology (Ujvari et al., 2014)
Fencing	Fencing has been used as part of wildlife management in different contexts with varying degrees of success. (Poor et al., 2014, Phillips et al., 2012)	Provides a barrier to DFTD	Expensive Chance that incursion of diseased animals might occur Interfering with the ecosystem and natural flow of native animals in and out of the area Need to monitor for inbreeding and overpopulation

1.16. DFTD vaccine development

1.16.1. Rationale for DFTD vaccine development

The highly conserved DFTD cell morphology and genotype lends itself to vaccine development (Woods et al., 2007) as does the demonstration that DFTD cells are immunogeneic (Kreiss et al., 2015). Likewise, evidence that devils have effective T cell function and the ability to mount an antibody response to immunisations (Kreiss et al., 2015, Brown et al., 2011) support vaccine research. Finally, the ability to up-regulate MHC-I on DFTD cells in vitro provides scope for vaccine development with the likelihood that MHC expression of foreign antigen will establish allorecognition (Siddle and Kaufman, 2012).

The development of a protective vaccine against DFTD would allow a vaccination protocol to be included as part of the Save the Tasmanian Devil Program's "wild devil recovery" project (DPIPWE). The aim of the project is to release devils from the captive insurance population and Maria Island (home to a translocated DFTD-free devil population) to augment wild populations that have been decimated by DFTD (Samantha Fox pers. comms 2016). It is also conceivable that populations of wild devils could be effectively vaccinated given that approximately 80% of devils should enter traps (Beeton and McCallum, 2011).

Although development of a protective DFTD vaccine is challenging, the progress made to date is encouraging and has advanced the understanding of this cancer and the devil's immune system. Transmissible cancers and marsupial immunology are two unique fields of research. Exploring their underlying features provides further insight into the immune escape mechanisms of transmissible cancers and the immune systems of marsupials that may have applications beyond the more immediate goal of DFTD vaccine development.

1.16.2. Cancer vaccines

Anti-cancer immunisation is a complex, multifactorial field and the optimal combination of antigens and adjuvants for different cancers is under active investigation (Melero et al., 2014). The antigenic basis of a vaccine requires a choice between whole tumour cells and isolated tumour peptides. Whole tumour cell vaccines utilise an array of tumour associated antigens (TAA's) containing epitopes for both cytotoxic T cells and T helper cells. The simultaneous

presentation of both MHC-I and MHC-II restricted antigens theoretically provide the advantage of generating a stronger overall anti-tumour response (Chiang et al., 2010). The alternative approach is to use isolated tumour peptides. A primary benefit of this method is that the risk of tumour development due to administration of whole cell derived preparations is eliminated. The drawbacks of peptide only vaccines include the difficulty of identifying appropriate antigens, and the increased risk of tumour escape with single epitopes (Chiang et al., 2010).

Some studies show better clinical outcomes using whole cell tumour vaccines compared to defined antigen (Neller et al., 2008). Since DFTD research to date has relied on the whole tumour cell preparations (Brown et al., 2011, Kreiss et al., 2015) an overview of this approach is provided here. Whole tumour cell vaccines require the cells to be made non-viable to prevent tumour inoculation developing out of the vaccine. Similarly, live tumour cells are often poorly immunogeneic and some can suppress immune responses (Chiang et al., 2010). These issues are addressed by using necrotic or apoptotic tumour cells. Necrosis is traumatic cell death due to injury and differs to apoptosis, also known as programmed cell death, which is a regulated and controlled process. There are numerous methods to achieve these states, all of which can enhance the immunogenicity of the cells in the process.

The most common method to induce necrosis of tumour cells is freeze-thaw cycles. Necrotic cells release heat shock proteins (HSPs) which are involved in antigen binding and presentation to the immune system. This makes them potentially useful adjuncts to cancer vaccines (Chen et al., 2009). Gamma irradiation results primarily in apoptotic cell death, with necrosis less likely (Baskar et al., 2012). High dose gamma irradiation of a variety of tumour cells has been associated with increased immunogenicity due to increased surface MHC-I expression (Reits et al., 2006); increased expression of surface MHC class I/II antigens and ICAM-I molecules (Chiriva-Internati et al., 2006); and cell surface translocation of calreticulin which promotes phagocytosis (Obeid et al., 2007). Radiation dosages in these studies ranged from 10 to 25 Gy and the expression of surface molecules and/or antigens increased with the dose. Single gamma irradiation doses of up to 80 Gy (Driessens et al., 2004, Zilberberg et al., 2011) have been used to induce apoptosis of tumour cells prior to their inclusion in cancer vaccines.

Tumour cell lysates have been described as ideal sources of a variety of TAA's associated with MHC-I and MHC-II molecules (Gonzalez et al., 2014). They are simple to produce and

methods include UVB radiation and hyperthermia treatment (Chiang et al., 2015). Sonication is another method for cell lysate production and acts by disrupting cell membranes with pulsed high frequency sound waves.

Increased immunogenicity of tumour cells is provided not only by inducing necrosis and/or apoptosis, but also by *in vitro* stimulation with cytokines. This can result in the increased expression of MHC-I, ICAM-I, ICAM-II and VCAM-I molecules, and possibly other molecules required for T cell activation (Guo et al., 1997). One disadvantage is the potential for cytokine incubation to up-regulate inhibitory molecules e.g. PDL1 on the tumour cell surface (Flies et al., 2016). Finally, the combination of gamma irradiation and cytokine incubation of tumour cells prior to their administration as a vaccine has been shown to have an additive effect with respect to the up-regulation and expression of cell surface antigen (Santin et al., 1996).

Adjuvants enhance the immune response to antigen and are frequently included in cancer vaccines due to the poor immunogenicity of tumour cells (Guo et al., 2013). The appropriate response elicited by a cancer vaccine is activation of cytotoxic T lymphocytes and IFN-γ-producing type 1 T helper cells (Th1) (Melero et al., 2014). A skewed response toward type 2 T helper cells (Th2) which generate a primarily humoral immune response is generally considered undesirable in cancer immunity. Adjuvants can target immune cells, influence the Th1-Th2 response, stimulate cytokine production and may reduce the required number of injections (Lefeber et al., 2003). Various adjuvant types e.g. oil emulsions, toll like receptor (TLR) agonists, and immune stimulatory complexes (ISCOMs) are often used in combination since single adjuvants tend not to result in clinically relevant anti-tumour activity (Melero et al., 2014). The interest in TLR agonists for cancer immunotherapy and cancer vaccine adjuvants, and the confirmation that devils have functional TLR's has seen current research focus on TLR agonists as useful adjuvants for DFTD vaccines (Patchett et al., 2015).

1.16.3. DFTD vaccine research to date

Immunisation trials have been carried out on a number of captive devils to date. The small sample size is a recurrent limiting factor but this has not prevented notable advances in DFTD vaccine development. Most important has been the demonstration that induction of an immune

response against DFTD cells is possible. This is noteworthy for a tumour characterised by its successful immune escape mechanisms.

A trial in 2011 in which two devils were immunised with four monthly doses of irradiated DFTD cells and montanide, an oil-based adjuvant, did not produce humoral or cell mediated immune responses against DFTD (Brown et al., 2011). However, subsequent trials on a total of eight devils using either freeze-thawed, irradiated or sonicated DFTD cells resulted in antibody and cytotoxicity responses in most devils (Kreiss et al., 2015). These cell preparations included montanide alone or in combination with CpG (a TLR agonist) as adjuvants. It was postulated that the inclusion of CpG to activate innate immune cells promoted the immune responses.

The humoral immune responses in this study were measured by detecting serum IgG antibody using ELISA, flow cytometry and western blots. Cytotoxic responses were assessed with a radioactive chromium release assay. It was thought unlikely the observed cytotoxic responses were mediated by CD8+ T cells since the DFTD cells in the assay did not express MHC-I. However, if IFN-γ was produced during the assay culture, this could up-regulate MHC-I expression and promote CD8+ T cell activity. An alternative explanation for the cytotoxicity was NK killing. Since there were no cytotoxic responses in non-immunised devils, spontaneous NK cell activity is unlikely. However, activation of NK cells may have occurred as a result of immunisation and translated to *in vitro* cytotoxic activity. One devil in the study resisted a DFTD challenge after demonstrating an antibody response, although it succumbed to a second challenge. The authors suggested other immunisation approaches could include DFTD cell modification to up-regulate MHC-I expression, consideration of alternative adjuvants, and isolation of tumour associated antigens.

1.16.4. Koala chlamydia vaccine research

As mentioned previously, there are parallels between devil and koala immunology. This applies particularly because research in both fields is directed toward developing protective vaccines against diseases that are threatening these iconic marsupials. With respect to the vaccine development, there are obvious differences between the target pathogens i.e. transmissible cancer cells of Schwann cell origin compared to intracellular bacteria which primarily infect mucosal tissue. Another disparity is the knowledge base for chlamydia immunobiology which

reflects its importance in human health. Even so, a preventative chlamydia vaccine for people is still not available. The immune response against chlamydia infection requires both T cell (CD4+ Th1 cells in particular) and B cell activity demonstrating that an effective vaccine needs to target both the cell mediated and humoral arms of the immune response (Brunham and Rey-Ladino, 2005, Hafner et al., 2014, Khan et al., 2016). Presumably T cell immunity is of primary importance for anti DFTD activity, and the role of humoral immunity is uncertain.

It is perhaps most useful to compare the immunisation protocols and testing of immune responses for devils and koalas. This is particularly true because the lack of available immunological reagents hampers the assessment of responses in both species. The vaccine preparation for koalas has relied on the recombinant chlamydia major membrane outer protein (rMOMP) as the antigen. Adjuvants have varied in the trials and include aluminum hydroxide gel, TiterMax Gold (which caused abscesses), a combination of poly I:C, polyphosphazene and host defence peptide, and Immune stimulating complex (ISC Pfizer/Zoetis) (Carey et al., 2010, Khan et al., 2014, Waugh et al., 2015, Waugh et al., 2016). Schedules have comprised a single dose, two doses at monthly intervals, and, more often, three doses at monthly intervals. Subcutaneous injection has been the usual route of administration but the response obtained by the intranasal route was also assessed (Waugh et al., 2015).

Methods to measure the humoral immune response required the preparation of anti-koala IgG. Serum IgG antibodies have been detected via ELISA using host specific C. pecorum MOMP or whole chlamydia “entire bodies” as antigen. Epitope specificity has been explored with PepScan methodology (Waugh et al., 2016). *In vitro* neutralisation assays are also employed (Carey et al., 2010, Waugh et al., 2015). Cell mediated immune responses were addressed by assessing total peripheral blood mononuclear cell proliferation, and CD4 proliferation in response to stimulation with chlamydia antigen (Waugh et al., 2015, Mangar et al., 2016). A chlamydia vaccine trial on 30 free-ranging koalas was carried out in 2013-14 (Waugh et al., 2016). Despite the differences in target pathogens and availability of some reagents, there is considerable scope for aspects of koala immunology/ chlamydia vaccine development and devil immunology/ DFTD vaccine development to be applied to each other.

1.17. Ecological impacts of a declining devil population

The devil is Tasmania's top order land predator and as such is a highly interactive keystone species. Consequently, its decline is expected to have significant deleterious impacts on Tasmania's ecosystem by advantaging feral predators. Changes in feral cat behaviour and possibly their increased abundance have been reported (Fancourt et al., 2015, Hollings et al., 2014). Devils may have prevented fox incursions in Tasmania from establishing in the past and thus protected the state's native fauna from the devastating effects foxes have had on the Australian mainland (Hawkins et al., 2006). Devils are specialised scavengers and the expected increase in carrion in the environment due to declining devil numbers could favour alternative scavengers (e.g. forest ravens), further disrupting the ecosystem balance (McQuillan, 2009). Thus, the devils' role in maintaining a healthy Tasmanian ecosystem is critical. Declining devil populations are already having measurable effects, and the extinction of the species in the wild would have profound consequences.

1.18. Final remarks

DFTD is a unique cancer that has developed strategies to avoid the devil's immune response and capitalised on the biting behaviour of devils to allow transmission between individuals. It has resulted in the suffering of countless devils and is having serious implications for Tasmania's ecosystem. Nonetheless, DFTD has provided unique opportunities to study transmissible cancers, including mechanisms of cancer cell transfer and immune escape. Likewise, it has necessitated the advancement of Tasmanian devil immunology.

While there is evidence of compensatory reproductive responses to population decline, and suggestion of genetic selection in response to DFTD, the future for the wild devil is not guaranteed. According to the literature, prior devil population bottlenecks have not been due to disease, and the likelihood of the species recovering from the DFTD epidemic unassisted is untested. As such, attempts to preserve the wild devil population through human intervention in the form of well-considered management plans and vaccine development are warranted.

DFTD vaccine research brings together the unusual fields of transmissible cancers and marsupial immunology. A deeper understanding of tumour immune escape mechanisms along with improved knowledge of a unique species' immune system is likely to have relevance

beyond DFTD and devils. Securing the future of the wild Tasmanian devil and by extension the Tasmanian ecosystem is reason enough to pursue the development of a protective DFTD vaccine. It is opportune if the results have broader applications.

Aims of thesis

Aim 1: To measure the peripheral blood T lymphocyte subset populations and serum levels of IgM and IgG in healthy devils, and analyse the effects of age, sex, season and DFTD status on each of these.

T lymphocyte subsets and immunoglobulins provide useful indicators of immune competence, and have been assessed in the lymphoid organs of healthy devils and those with DFTD. The effects of DFTD on haematology and serum biochemistry parameters in the Tasmanian devil have been published. However, DFTD's effects on the immunological components of the devil's peripheral blood require exploration.

Aim 2: To look for the presence of an immune response against DFTD in wild Tasmanian devils in the form of serum anti-DFTD IgG antibodies and/or infiltrating T lymphocytes in tumour biopsies.

DFTD is an aggressive cancer with most devils dying within 6 to 12 months of clinical signs first appearing. The literature suggests that DFTD always escapes the devil's immune system and that death is the inevitable consequence of infection. These assumptions were revisited for this aim. Devils that had reportedly undergone tumour regression were included in this study.

Aim 3: To immunise captive devils with irradiated DFTD cells modified to express surface MHC-I, and assess the devils' subsequent immune responses and the consequences of a live DFTD cell challenge.

DFTD vaccine development has been underway since 2006. The conserved morphology of the DFTD cell and the devil's demonstrated ability to mount immune responses against DFTD cell preparations make vaccine development a reasonable goal. The epigenetic down-regulation of the major histocompatibility complex class I molecule (MHC-I) is considered a principle mechanism by which the DFTD cells escape the devil's immune response. This down-regulation is reversible and DFTD cells incubated with the cytokine interferon gamma (IFN-γ) express surface MHC-I. These MHC-I^{+ve} cells are expected to be immunogeneic and provide further scope for vaccine development.

Aim 4: To assess the immune responses to the immunisation protocol used in Aim 3 on 19 devils prior to their wild release. To also assess the effects age and sex had on the responses.

The state government's Save the Tasmanian Devil Program's "wild devil recovery project" allowed for the immunisation protocol used in a trial from Aim 3 to be carried out on captive held devils prior to their wild release. The relatively large sample size allowed the immune responses to be more robustly assessed than had been possible in previous trials. Follow up monitoring trips would allow the duration of immune responses to be assessed in devils that remained in the vicinity of the release site.

Aim 5: To determine if immune cross-recognition of DFT1 and DFT2 occurs in devils by assessing the serum IgG antibody responses to DFT2 in wild and immunised devils that had antibody responses to DFT1.

While this thesis was underway a second transmissible cancer affecting Tasmanian devils was discovered. This was named DFT2 and bears similar morphological features to DFT1 (the first DFTD). However, there are profound differences between the two tumours at the molecular level, and genetic analyses confirmed that DFT2 arose independently to DFT1. This aim was to explore whether similar antigens occur on the two tumours, allowing for immune cross-recognition by devils.

Chapter 2

General materials and methods

Chapter 2. General Materials and Methods

2. 1. Materials

Table 2.1. Reagents (not including antibodies).

Reagent name	Supplier	Catalogue number
Antibiotic-Antimycotic	Life Technologies	15240-062
Amniomax	Life Technologies	11269-016
Bovine serum albumin (BSA)	Sigma-Aldrich	A7906-100G
Cell Trace ™ Violet Cell Proliferation Kit	Life Technologies	C34557
Concanavalin A (Con A)	Sigma-Aldrich	C 7275
CpG oligonucleotide 1585 (CpG 1585)	GeneWorks	1141231
CpG oligonucleotide 2395 (CpG 2395)	GeneWorks	1141232
Diamidino -2-Phenylindole, Dihydrochloride (DAPI)	ThermoFisher	D1306
Diaminobenzidine (DAB) solution	Dako	K3466
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	D-5879
Envision Plus System – HRP labelled polymer Anti-Mouse	Dako	K4001
Envision Plus System – HRP labelled polymer Anti-Rabbit	Dako	K4003
Eosin	Sigma-Aldrich	2853
Ethanol	Sigma	E-7023-1L
Foetal bovine serum (FBS)	Bovogen	SFBSF7
Formalin, 10% neutral buffered	Fronine	ENNJJ019
GlutaMAX™	Life technologies	35050-061

Reagent name	Supplier	Catalogue number
Haematoxylin	Sigma-Aldrich	517-28-2
Hydrochloric acid (HCl)	VWR	VWRC20255.290
Hydrogen Peroxide	Sigma-Aldrich	676-3
Interferon gamma (IFN-γ) recombinant devil	WEHI	not a commercial product
ISCOMATRIX™	CSL Ltd, Victoria, Australia	not a commercial product
ISOTHESIA® Isoflurane	Henry Schein, Northgate, Australia	050031
Methanol	Merck	4.10232.2500
Protein block serum free	Dako	X0909
Phosphate buffered saline (PBS) tablet	OXOID	BR0014G
Poly(I:C)	Sigma-Aldrich	42424-50-0
Propidium iodide (PI)	Sigma-Aldrich	P4170
RNAlater®	Qiagen	1018087
Roswell Park Memorial Institute 1640 medium (RPMI)	Life Technologies	11875-093
Sodium azide (NaN3)	BDH AnalaR	301112G
Sodium bicarbonate (NaHCO3)	Sigma-Aldrich	S5761
Target Retrieval Solution	Dako	S1699
TMB SureBlue™ TMB Microwell Peroxidase Substrate	KPL	52-00-01
Trypan blue (TB)	Chroma	11661
Tween®20	Sigma	P1379

Table 2.2. Antibodies used for ELISA, flow cytometry and immunohistochemistry.

Antibody	**Supplier**	**Catalogue number or ID ± concentration if not a commercial antibody**
IgG goat anti-mouse Alexa Fluor® 488	ThermoFisher	A-11001
IgG goat anti-rabbit Alexa Fluor® 647	ThermoFisher	A32733
IgG goat anti-mouse Alexa Fluor® 647	Life Technologies	A21235
CD3 polyclonal rabbit anti-human	Dako	A0452
CD4 monoclonal mouse anti-devil	Walter and Eliza Hall Institute	8C9 3.4 mg/ml
CD4-AF647 monoclonal mouse anti-devil	Walter and Eliza Hall Institute	7H9
CD8 monoclonal mouse anti-devil	Walter and Eliza Hall Institute	10E8 4 mg/ml
MHC-II anti-human HLA-DR	Dako	M0746
CD79b rat anti-mouse	AbD Serotec	MCA2209
Periaxin rabbit anti-human	Sigma-Aldrich	HPA001868
Beta 2 microglobulin (β_2m) mouse anti-devil	Provided by Hannah Siddle	13-34-45
IgG monoclonal mouse anti-devil	Walter and Eliza Hall Institute	A4D1 2mg/ml
IgM monoclonal mouse anti-devil	Walter and Eliza Hall Institute	46-11-8B6 2 mg/ml
mouse IgG2b isotype	LifeSpan BioScience	LS-C149303
Polyclonal Goat Anti-Mouse Immunoglobulins/HRP	Dako	P0447
Polyclonal Goat Anti-Rabbit Immunoglobulins/HRP	Dako	P0448

Table 2.3. Disposables.

Disposables	Supplier	Catalogue number
Cell culture cluster, 96 well round bottom with lid	Corning	3799
Centrifuge tube 10 ml	Thermo Fisher	LBSCT1003X
Centrifuge tube 15 ml	Corning Inc	430791
Centrifuge tube 50 ml	Corning Inc	430829
Clot activating tube 9 ml	Greiner Bio-one	455092
Costar® Assay Plate, 96 well clear, flat bottom	Corning	9018
Cryogenive vial 2 ml	Corning	430488
Lithium heparin tube 9 ml	Greiner Bio-one	455084
Microcentrifuge tubes 1.5ml	Sigma Aldrich	80-1500
Microscope slides Flex IHC	Dako	K8020
Needle 21 G x 1" hypodermic	Terumo	2125RL
Pipette tips 0.1 – 20 µl	Edwards Instruments Co.	1151-965-008
Pipette tips 02 – 200 µl	Edwards Instruments Co.	1030-260-000
Pipette tips 50 – 1000 µl	Edwards Instruments Co.	1057-965-018
Punch biopsy 4 mm	Kai Medical	1072022
Syringe 10ml	Terumo	SS+10S
Syringe 3 ml	Terumo	SS+03S
Syringe 5 ml	Terumo	SS+05S
Tissue culture flask T25	Corning	430639
Tissue culture flask T75	Corning	430720
Transfer pipette 3 ml	LabServ	LBSCNF108

Table 2.4. Equipment.

Equipment name	Supplier
Anaesthetic machine Stinger ST 205	AAS-Advanced Anaesthesia Specialists
Autoprocessor ASP 200S	Leica
BD CANTO II flow cytometer	Becton Dickinson
Centrifuge Allegra ® X-12	Beckman Coulter
Class II biological safety cabinet S@femate 1.2	Bio Cabinets
Incubator Heraeus	Function Line
Light microscope	Olympus
Microscope camera	Leica
Neubauer chamber (cell counts)	Sigma-Aldrich
Portable pulse oximeter	VetQuip
SpectraMax M2 Multi-Mode Microplate reader	Molecular Devices
Ultrasonic cell disruptor	Misonix Inc.
Varian Clinac 23-EX linear accelerator (Holman Clinic, Royal Hobart Hospital)	Varian Medical Systems Inc.

Solutions prepared in the laboratory:

Blocking buffer

200 ml PBS + 2 ml FBS + 0.2 g BSA + 1 ml 5% azide

Carbonate buffer

2.1 g $NaHCO_3$ in 250 ml Milli-Q ® water, pH 8.2

Complete Medium (CM)

RPMI 1640 supplemented with 10% FBS, 1% GlutaMAX™ and 1% Antibiotic-Antimycotic

Con A supernatant ("Con A sup")

supernatant of Tasmanian devil PBL's stimulated with 5 µg/ml Concanavalin A for 48 hours in culture medium

FACS buffer

PBS (prepared from tablets) + 1% BSA + 0.1% NaN3

Freezing medium (2x concentrate)

20 ml DMSO + 80 ml FBS (i.e. 20% DMSO)

Phosphate Buffered Saline (PBS)

PBS tablets x 5 in 500 ml Milli-Q ® water, stirred with a magnetic stirrer

Stop solution

1 Molar HCl

Washing buffer

500 µl Tween + 1 L PBS

2.2. Methods - Laboratory

2.2.1. Immunohistochemistry

Standard haematoxylin and eosin (HE) and immunohistochemical staining were performed on three-micrometre paraffin sections from tumour tissues fixed in 10% neutral buffered formalin, embedded in paraffin wax and sectioned (3 μm) onto 3-aminotriethoxysilane (Sigma-Aldrich) coated slides. HE staining was performed at Pathology South laboratory at the Royal Hobart Hospital.

Primary antibodies and their dilutions for immunohistochemistry were as follows:
anti-devil CD4 (1:50)
anti-devil CD8 (1:100)
anti-mouse CD79b (1:400)
anti- human HLA-DR (MHC-II) (1: 40)
anti-human CD3 (1:300)
anti-human periaxin (1:300)

The IHC method used was that described in (Howson et al., 2014). In brief, sections were deparaffinized in a histology oven at 60 ºC for 15 minutes followed by two five-minute washes in xylene and rehydration through successive graded ethanol solutions and washed for five minutes in distilled water. Heat-induced antigen retrieval was carried out in citrate buffer, pH 6 (Dako, Carpinteria, CA) using an electric pressure cooker at medium heat for ten minutes, followed by a 20-minute cooling period at room temperature. The slides were incubated with serum-free blocking solution (Dako) for 30 minutes and then incubated with the primary antibody for one hour. The slides were then immersed in 3% hydrogen peroxide in phosphate-buffered saline (PBS) for ten minutes at room temperature to quench endogenous peroxidase activity. The anti-mouse or ant-rabbit EnVision System, HRP, (Dako) was used for signal detection. Liquid DAB1 Substrate Chromogen System (Dako) was added to each slide and incubated for ten minutes. The sections were counterstained with hematoxylin and mounted.

Assessment of the biopsy samples and staining for Chapter 4 was performed by a veterinary pathologist, and for Chapter 5 by a pathology registrar (in human pathology).

A light microscope (Olympus-BX50) coupled with a camera (Leica-DFC320) was used for visualization and acquisition of the images.

2.2.2. Serum separation

Blood samples were collected into clot activating tubes. After a minimum of 30 minutes (to allow clot formation) the tubes were centrifuged at 1000 g for 10 minutes. The serum was pipetted off and aliquoted and stored at -20 °C (short term) or -80 °C (long term).

2.2.3. Peripheral blood lymphocyte (PBL) isolation

Reagents were warmed to room temperature prior to use, and the following steps carried out under sterile conditions. Blood samples collected into lithium heparin tubes were diluted 1:1 with PBS. Up to 8 ml of the diluted blood was then overlayed on 4 ml Histopaque 1077® in 15 ml tubes. Tubes were centrifuged at 400 g for 30 minutes, with no brake. The mononuclear cells at the interface was pipetted off with a 3 ml transfer pipette. Cells were washed twice in RPMI 1640, centrifuged at 200 g for 10 minutes each time. The pellet was resuspended in complete medium and a cell count performed on a Neubauer chamber.

2.2.4. Cell culture of established cell lines

Three different DFT1 cell lines (C5065 = strain 3, year of origin 2007; 1426 = strain 2, year of origin 2005; and Ed = strain 2, year of origin 2012) were maintained in T75 flasks with Complete Medium.
Two different DFT2 cell lines (Snug/ TD500, year of origin 2014; and TD549, year of origin 2015) were maintained in T75 flasks with Complete Medium + 20% Amniomax.

All cells were kept at 35 °C in a humidified atmosphere of 5 % CO_2 in air.

2.2.5. Up-regulation of surface expression of MHC-I on DFTD cells

DFTD cells were treated with recombinant devil INF-γ (50 ηg/ml added to CM) and incubated for 24 hours at 35 °C in a humidified atmosphere of 5% CO_2 in air. The MHC-I surface expression was confirmed by flow cytometry (see 2.2.7).

2.2.6. Cell counts

Counts of tumour cells and peripheral blood lymphocytes were performed with a Neubauer Chamber and a light microscope using the 200 or 400 x objective. 10 µl of sample was mixed with 10 µl trypan blue and pipetted onto the counting chamber. Cells in the central 25 squares were counted. Total cell concentration (TCC) was calculated using the formula:

TCC/ ml = number of cells counted x (25/ number of squares counted) x dilution factor x 10^4

2.2.7. Flow cytometry

CD4 staining on devil PBL's

PBL's were washed and resuspended in FACS buffer at 10^6 cells/ml. 200 µl of sample was incubated with mouse anti-devil CD4 AF647 (1:4,000) for 30 minutes on ice. Cells were washed twice, resuspended in DAPI (3 µM) and analysed with the Canto flow cytometer.

To confirm surface expression of β_2m (a component of MHC-I) on DFTD cells:

INF-γ treated cells were washed 3 times with FACS buffer before each of the following steps. Cells were incubated with mouse anti-devil β_2m (1:200) for 30 minutes on ice. Cells were then incubated with goat anti-mouse IgG Alexa Fluor® 647 (1:1,000) for 30 minutes on ice. They were re-suspended in 200 µl FACS buffer containing 3 µM PI to allow gating of dead cells, and analysed by flow cytometry (BD Canto II).

To detect anti DFTD IgG antibody:

DFTD cells were washed 3 times with FACS buffer before each of the following steps. Serum samples were diluted 1:50 with FACS buffer and incubated with approximately 2 x 10^5 MHC-I^{+ve} DFTD cells/ well and separately with 2 x 10^5 MHC-I^{-ve} DFTD cells/ well in 96 well plates for one hour on ice. Cells were then incubated with a monoclonal mouse anti-devil IgG antibody (1:200) for 30 minutes on ice. Finally, cells were incubated with a fluorochrome labelled goat anti-mouse IgG Alexa Fluor® 647 (1:1000) for 30 minutes on ice. They were resuspended in 200 µl FACS buffer containing 3 µM PI to allow gating of dead

cells, and analysed by flow cytometry (BD Canto II). Cells labelled with secondary and tertiary antibodies only gave no background fluorescence. The mouse IgG2b isotype (Bioscience) controls showed the same MFI as the no-serum controls.

2.2.8. Cytotoxicity assays

DFTD cells from culture were washed twice with PBS (500 g, five minutes), suspended in PBS and stained with cell trace violet (CTV) at 2 µl CTV per 10^6 cells, and incubated for 20 minutes at 35 °C in a humidified atmosphere of 5% CO_2 in air. The CTV was quenched with 10 ml complete medium (CM) for five minutes at 35 °C. A cell count was performed and the cells re-suspended in CM to a concentration of 10^5 cells/ml.

PBL's were separated (2.2.3), counted and re-suspended in CM at 10^7 cells/ ml or 5 x 10^6 cells/ ml. In a 96 well round-bottomed plate (Corning), 200 µl of the PBL suspension was added to wells in row A in triplicate or quadruplicate. 100 µl of CM was added to the wells in rows B to G. The PBL suspension was titrated down from row A to row G leaving a final volume in each well of 100 µl.

100 µl of the DFTD cell suspension was then added to each well so that PBL:DFTD ratios started at 100:1 in row A, down to 1:1 in row G. (If only 5 x 10^6 PBL/ ml were available, the dilution started at 50 PBL:1 DFTD cell). Row H contained DFTD cells only to measure control well cell death. The plate was incubated overnight at 37 °C in a humidified atmosphere of 5% CO_2 in air.

The following morning, assays were centrifuged at 1500 rpm for three minutes, the supernatants pipetted off and stored at -80 °C, and the cells re-suspended in 200 µl propidium iodide (PI) diluted 1:500 with FACS buffer. The plates were then run on the BD Canto for flow cytometry analysis. The PBL's were gated out by selecting the CTV positive cells. The percentage of PI positive CTV positive cells (i.e. dead DFTD cells) was recorded.

Cytotoxicity = (% dead cells - % control well cell death)/(1- % control cell well death)

Cytotoxicity curves were plotted using GraphPad Prism.

2.2.9. Immunisation preparation

C5065 cell line was used for all immunisations

MHC-I up-regulation

For immunisations used in Chapter 5 Trial 1:
Surface expression of MHC-I was up-regulated on DFTD cells by incubation with 10% cytokine rich conditioned medium ("Con A sup") plus recombinant devil IFN- γ at 1:10,000 dilution in culture for 24 hours at 35 °C in a humidified atmosphere of 5 % CO_2 in air.

For immunisations used in Chapter 5 Trial 2, and Chapter 6:
MHC-I was up-regulated in DFTD cells by incubation with IFN-γ at 1:5,000 dilution in culture for 24 hours at 35 °C in a humidified atmosphere of 5 % CO_2 in air.

MHC-I expression was confirmed with flow cytometry with mouse anti-devil β_2m antibody staining (2.2.7).

Sonicated cell vaccine preparation

The MHC-I^{+ve} DFTD cells were counted, suspended in PBS at 2 x 10^7 cells/ ml, and sonicated with four ultra-sonic cycles using 50% power using an ultrasonic cell disruptor – Misonix Inc., then aliquotted at 1 ml per cryovial, and stored at -80 °C.

Irradiated cell vaccine preparation

The MHC-I^{+ve} DFTD cells were suspended in CM and placed in 1.8 ml cryovials and irradiated. Radiation treatment comprised two doses of 40 Gy of gamma radiation 24 hours apart using a Varian Clinac 23-EX linear accelerator – Varian Medical Systems Inc. The cells were kept in the crovials at 35 °C in a humidified atmosphere of 5% CO_2 in air for the 24 hours in between doses.
Following radiation, the cells were pooled, centrifuged (500 g for 5 minutes), and the pellet resuspended in CM. They were counted and 2 x 10^7 cells in 0.9 ml CM were mixed with 0.9 ml of 2 x freezing medium and placed in 1.8 ml cryovials and stored at -80 °C.

Adjuvants

The adjuvants added to each vaccine for the trials in Chapters 5 and 6 were:
50 µl ISCOMATRIX™
50 µg CpG 1585
50 µg CpG 2395
100 µg poly(I:C)

2.2.10. Cytogenetic analysis

This procedure was performed at the Animal Health Laboratory, Prospect, Tasmania, 7250.

Before harvesting cells from culture, 0.1 ml of demecolcine at 10 µg/mL (Sigma-Aldrich) was added to each culture and incubated for four hours. The cells were centrifuged for ten minutes at 1000 rpm. Supernatant was discarded and the cell pellet was slowly resuspended in 7 ml of hypotonic 0.075 M KCl and placed in a water bath at 37 °C for 18 minutes. 2 ml of chilled Carnoy's fixative (3:1 ratio of methanol to acetic acid) was added and the tubes centrifuged for ten minutes at 1000 rpm. After removing the supernatant the pellet was gently resuspended in Carnoy's fixative and stored at 4 °C overnight. The following day the cells were spun and resuspended in three changes of fresh fixative. The cells were then resuspended in enough fixative to form a milky suspension. This suspension was dropped onto clean, frozen microscope slides from 10 cm to ensure chromosome spread. Slides were allowed to dry and then placed in an oven at 57 °C for three days before banding. G-banding was conducted by treating slides with a 0.15 % solution of trypsin for up to 30 seconds before staining with Leishmann's stain for 2½ minutes, then mounting with Leica mounting medium (Leica Microsystems) for analysis.

G-banding analysis was performed using a Leica DM 2000 microscope (Leica Microsystems) and photographed with a Leica DFC 420 C camera (Leica Microsystems). Karyotypes were made using VideoTest Karyo 3.1 (VideoTest). 50 Metaphases were analysed for each sample.

2.2.11. Software/ Programmes

GraphPad Prism version 6 for Mac OS X, GraphPad Software, La Jolla, CA, USA, www.graphpad.com

Kaluza version 1.5 Flow Cytometry Analysis Software

R statistical software

2.3. Methods – Tasmanian devil housing and handling

2.3.1. Animal ethics

The research was carried out in compliance with and with approval from the University of Tasmania's Animal Ethics Committee under permit numbers:
A0014976; A0014599; A0013685; A0012513

2.3.2. Housing of captive devils

The Tasmanian devils were housed in secured shelters in accordance with the guidelines of the Department of Primary Industries, Parks, Water and Environment of Tasmania (DPIPWE). All experimental methods were performed in accordance with the University of Tasmania guidelines.

2.3.3. Blood collection

From wild and captive devils that could be handled:
Devils were handled in the sack and between 2 and 5 ml (wild devils) and up to 10 ml (captive devils) of blood was collected from the jugular vein with a 21 G hypodermic needle and a 5 or 10 ml syringe.

From captive devils that couldn't be handled conscious:
Devils were anaesthatised as described below. They were placed in ventral recumbancy and up to 10 ml blood collected from the jugular vein.

2.3.4. Tumour biopsies

Samples were collected using 4 mm biopsy punches (Kai). Samples were divided and fixed in 10% neutral buffered formalin and/or RNAlater ®.

2.3.5. Tumour fine needle aspirates (FNA's)

Tumour aspirates were collected with a 21G or 23G hypodermic needle and 5 ml syringe. For cytology, these were expressed on to glass microscope slides, air dried and stained with May Grunwald-Giemsa stain (Animal Health Laboratory, Prospect, 7250).

Aspirates collected for immunocytochemistry analysis were placed directly into 1ml vials of 4% paraformaldehyde, and after approximately 10 days (on return from the field) were centrifuged and resuspended in phosphate buffered saline for long term storage.

Tumour aspirates for cell culture and karyotype were collected as described above and placed directly into 5 ml of transport medium (Complete Medium + 2% Antibiotic-Antimycotic).

2.3.6. General anaesthesia

General anaesthesia was induced with 5% Isoflurane (ISOTHESIA®) reducing to a maintenance concentration of 3% via a face mask. A pulse oximeter was clipped to the devil's ear to record heart rate and oxygen saturation. An assistant monitored the anaesthesia throughout the procedure.

Chapter 3

The effects of DFTD, age, sex and season on peripheral blood T lymphocytes and immunoglobulins in Tasmanian devils

Chapter 3. The effects of DFTD, age, sex and season on peripheral blood T lymphocytes and immunoglobulins in Tasmanian devils.

3.1. Introduction

The successful transmission of devil facial tumour disease (DFTD) as an allograft between Tasmanian devils raises many immunological questions about the disease, the devil's immune system and their interplay. As appropriate reagents become available these questions are being addressed. Peripheral blood T lymphocytes and immunoglobulins provide useful indicators of immune competence. These are commonly measured in humans and increasingly assessed in other species. The CD4:CD8 ratio has been used to provide an indication of immune competence in people and this has been particularly useful for identifying the effect of HIV on T cells (Margolick et al., 2006). Likewise, serum immunoglobulin levels can help gauge an immune response. Serum total protein is separated into albumin and globulins, with the globulin fraction further divided into alpha, beta and gamma globulins (see Appendix 1 for protein electrophoresis results of six healthy devils and six devils with DFTD). The gamma globulins include the immunoglobulins which are categorised as IgM, IgG, IgA, IgE and IgD.

Immune competence is affected by various parameters including cancer, age, sex and season. This has been explored by measuring the effects these parameters have on T lymphocytes and immunoglobulin levels in peripheral blood, most widely in humans but also other species including domestic pets (Heaton et al., 2002a, Heaton et al., 2002b) and wild animals (Mangar et al., 2016, Flies et al., 2015). The development of devil-specific antibodies binding CD4 and CD8 in formalin fixed tissue, and IgM and IgG in fresh and fixed tissue has allowed the identification of these components in the lymphoid organs of devils (Howson et al., 2014). While it is known that devils with DFTD have a lower total lymphocyte count than healthy devils (Peck et al., 2016), further study of T lymphocyte subset populations in peripheral blood has been hampered by a lack of reagents for fresh blood analysis and the logistical difficulty of separating peripheral blood lymphocytes (PBL's) from samples collected in remote field locations. Serum levels of IgM and IgG in healthy and diseased devils are also yet to be determined.

This study aimed to measure T lymphocyte subsets and IgM and IgG levels in peripheral blood samples collected from a DFTD affected population of wild devils over a 12 month period. As

the study was nearing completion, an anti-devil CD4 binding antibody became available for flow cytometry. Until then, however, devil specific CD4 and CD8 binding antibodies were available only for immunohistochemistry (IHC). To overcome the limitations of the available reagents and PBL separation, a novel method of fixing blood clots in formalin to preserve an ample number of PBL's for IHC staining and subsequent calculation of the T lymphocyte subset percentages was used. Indirect ELISA was the method employed for measuring levels of serum IgM and IgG.

Here the percentages of peripheral CD4+ and CD8+ T lymphocytes, and the relative levels of IgM and IgG found in healthy devils is reported. To determine if these values are altered by DFTD was a primary aim of this study and is also shown. The effects of age, sex and season on these peripheral blood components in healthy devils is evaluated and how the results compare with other mammalian species is discussed.

3.2. Materials and methods

3.2.1. Samples and sample collection

The blood samples were collected from wild devils (n=58) at a study site at West Pencil Pine in north west Tasmania (41°31' S, 145°46' E). The population is trapped at the same times each year to coincide with significant annual events in the devil population: February (summer, pre-breeding season); May (autumn, post-breeding season); August (winter, pouch young); and November (spring, late lactation) (Hamede et al., 2013). Samples for this study were collected over a 12 month period covering each of the four seasons. Devils over two years of age were classified as adults, and younger than two years as juveniles.

A diagnosis of DFTD was based on visual assessment of tumour location and appearance according to previously described classification methods (Hawkins et al., 2006). Tumour biopsies were collected for 17 of the 22 devils diagnosed with DFTD (see Chapter 2.3.4 for biopsy procedure). It was not possible to collect tumour samples from the remaining five devils due to the location of the tumour e.g. under the tongue or on the hard palate.

Blood (3-5 ml) was collected from the jugular vein with a 21 G needle and 5 ml syringe and transferred directly into clot activating tubes. The serum was pipetted off within five hours of

collection and the remaining blood clots placed in 10% neutral buffered formalin for between three and five days before processing.

3.2.2. Immunohistochemistry and validation experiments

Immunohistochemistry

See Chapter 2.2.1 for IHC staining procedures with haematoxylin and eosin (HE), mouse anti-devil CD4 and CD8 antibodies, rat anti-mouse CD79b, and anti-human MHC-II.

Validation experiments

This was a novel method and so three validation experiments were performed:

1. White cell identification (morphology) (n=5)

Preservation of cell morphology in the fixed blood clot sections for identification of neutrophils and lymphocytes was assessed. The standard method to obtain white blood cell differentials on devil blood samples is to perform manual counts on fresh blood smears. To confirm that cell identification in the fixed blood clot sections was adequate, blood samples were collected from five devils and each divided into EDTA and clot activating tubes. The five EDTA samples were sent to the Animal Health Laboratory (AHL), Department for Primary Industries, Parks, Water and Environment, Tasmania for manual differentials. These were performed on fresh blood smears stained with Diff-Quik (a Romanowsky stain). The blood clots were fixed and processed as described above and stained with HE. The number of lymphocytes and neutrophils out of 100 white blood cells was counted on these HE stained clots (manual count under 40 x objective). This count was repeated two or three times and the average taken. The results obtained from the blood smears and the HE stained clots were compared.

2. Flow cytometry compared to IHC method to assess percentages of CD4+ and CD8+ lymphocytes on human samples (n=5):

Antibodies binding human CD4 and CD8 are available for use on both fresh (for flow cytometry) and fixed (for IHC) human tissue. The conventional method for obtaining

percentages of CD4+ and CD8+ lymphocytes in human blood is with flow cytometry. To validate the IHC method for obtaining CD4+ and CD8+ lymphocyte percentages in peripheral blood, five human blood samples were collected and each divided into EDTA and clot activating tubes. The percentages of CD4+ and CD8+ lymphocytes were assessed on the EDTA samples by flow cytometry at the NATA accredited Pathology South laboratory at the Royal Hobart Hospital. The clots were formalin fixed for sectioning and stained with anti-human CD4 and CD8 antibodies at the same laboratory. Sections were examined under 40 x objective and the number of CD4+ or CD8+ lymphocytes out of 100 lymphocytes was counted for each section to acquire the percentage of CD4+ or CD8+ lymphocytes. The counts were repeated two or three times and the average taken. CD4+ lymphocytes were distinguished from CD4+ monocytes on the basis of morphology. The results obtained by the flow cytometry and IHC methods were compared.

3. Flow cytometry compared to IHC method to assess percentage of CD4+ lymphocytes on devil samples (n=4)

Towards the end of this study a mouse anti-devil CD4 antibody was developed for flow cytometry which allowed a third validation experiment to be performed.

Blood samples were collected from four devils and each divided into EDTA and clot activating tubes for processing as described above. Flow cytometry with the anti-devil CD4 antibody was performed on PBL's isolated from the EDTA samples. See Chapter 2 for PBL isolation (2.2.3) and flow cytometry staining (2.2.7). Formalin fixed clots were sectioned and stained with anti-devil CD4 antibody. Sections were examined under 40 x objective and the number of CD4+ lymphocytes out of 100 lymphocytes was counted for each section to acquire the percentage of CD4+ lymphocytes. This count was repeated two or three times and the average taken. The results obtained by the flow cytometry and IHC methods were compared.

3.2.3. Calculation of percentages of CD4+ and CD8+ lymphocytes for the wild devil samples

Fixed blood clot sections, each stained with either anti-devil CD4 or CD8 antibody were examined under 40 x objective and, as described above, the number of CD4+ or CD8+ lymphocytes out of 100 lymphocytes was counted for each section. This count was repeated

two or three times and the average taken to give the percentage of CD4+ or CD8+ lymphocytes present in each peripheral blood clot. Sections were also stained with the cross-reactive rat anti-mouse CD79b and anti-human MHC-II to identify B cells and antigen presenting cells (Kreiss et al., 2009). See Chapter 2.2.1 for CD79b and MHC-II staining.

3.2.4. Measurement of serum IgM and IgG in wild devils by indirect ELISA

Serum samples were diluted to 1:200,000 in carbonate buffer, and 100 µl of each sample pipetted into a single well of a Costar® Assay 96 well plate. The plate was covered in parafilm and kept at 4 °C overnight. The following morning, each well was washed with washing buffer three times then 200 µl of blocking buffer was added to each well and incubated at room temperature for a minimum of two hours. The blocking buffer was discarded and 100 µl of diluted IgM or IgG (IgM diluted at 1:500 with blocking buffer; IgG diluted at 1:200 with blocking buffer) added to each well and incubated at room temperature for one hour. Each well was washed with washing buffer three times. Goat anti-mouse horseradish peroxidase was diluted 1:2000 with blocking buffer and 100 µl added to each well for an hour incubation at room temperature. Each well was washed with washing buffer three times and TMB 100 µl per well was added and left for five to ten minutes depending on colour intensity before adding 100 µl of stop solution to each well. The plate was read at 450 nm on a SpectraMax Microplate reader. The sample results are reported as optical density (OD), and are relative to each other.

3.2.5. Statistics

Arcsine transformation of percentages of all cells (lymphocytes, neutrophils, CD4+ and CD8+ lymphocytes) was performed prior to all statistical analyses to normalise the distribution of the percentage values.

Student's paired t tests were performed for each of the validation experiments.

A three-way ANOVA followed by Tukey's posthoc analysis was performed to determine the effects of season, age and sex on each of CD4+ and CD8+ percentages, CD4:CD8 ratio, IgM and IgG levels, and IgM:IgG ratio in healthy devils.

Student's unpaired t tests were performed to compare healthy and diseased devils for each of CD4+ and CD8+ percentages, CD4:CD8 ratio, IgM and IgG levels and IgM:IgG ratio.

Two-way ANOVAs followed by Sidak's multiple comparisons tests were performed to determine the effects of DFTD and age on CD4+ and CD8+ percentages, CD4:CD8 ratio, IgM and IgG levels, and IgM:IgG ratio.

Arcsine transformation, Student's t tests and two-way ANOVAs were performed using GraphPad Prism version 6; and the three-way ANOVA and Tukey's posthoc analysis was performed using R statistical software.

3.3. Results

3.3.1. Tasmanian devils and blood samples

The number of devils sampled over the 12 month period, and their age, sex and DFTD status is summarized in Table 3.1.

Table 3.1. Numbers of devils sampled according to age, sex, DFTD status and season collected.

Total samples			DFTD-			DFTD+	
58			36			22	
	Juvenile		Adult		Male		Female
DFTD-	25		11		17		19
DFTD+	6		16		9		13
total	31		27		26		32
	November spring		February summer		May autumn		August winter	
DFTD-	8		10		9		9	
DFTD+	9		5		5		3	
	D-	D+	D-	D+	D-	D+	D-	D+
Juvenile	6	4	6	0	5	0	8	2
Adult	2	5	4	5	4	5	1	1
Male	4	3	5	3	3	2	5	1
Female	4	6	5	2	6	3	4	2
Juvenile male	3	2	4	0	1	0	4	0
Juvenile female	3	2	2	0	4	0	4	2
Adult male	1	1	1	3	2	2	1	1
Adult female	1	4	3	2	2	3	0	0

DFTD- and D- signify healthy devils without DFTD; DFTD+ and D+ are devils with DFTD; juvenile devils are < 2years old, adult devils are > 2years old

3.3.2. Validation experiments

1. White cell identification /morphology (n=5)

Cell morphology was adequately preserved in HE sections of formalin fixed blood clots to allow for identification of lymphocytes and neutrophils (Fig. 3.1a). To determine if this identification was reliable, the number of lymphocytes and neutrophils out of 100 cells was counted on HE stained fixed clots from blood samples of five devils. Fresh blood smears were made from the same five samples, and white blood cell differentials performed on these. Student's paired t tests were carried out to compare the overall results for lymphocytes and neutrophils obtained by each method. There was no significant difference between the results obtained by the two methods for the percentages of either lymphocytes ($p = 0.890$) or neutrophils ($p = 0.144$) (Table 3.2).

Table 3.2. Comparison of lymphocyte and neutrophil percentages obtained by two different methods for five individual devils.

	%Lymphocytes		%Neutrophils	
Devil	Blood smear	HE	Blood smear	HE
Barney	24	26 ± 2	68	63 ± 3
Aslan	35	49 ± 2	52	46 ± 2
Rose	35	30 ± 1	52	54 ± 2
Pansy	53	53 ± 2	45	35 ± 2
Smithy	55	41 ± 2	41	41 ± 3

The percentages of lymphocytes and neutrophils for each of five blood samples were obtained by two different methods. Manual white blood cell differentials were performed on fresh blood smears by the Animal Health Laboratory which provided the results. Formalin fixed blood clots were stained with HE and the number of lymphocytes and neutrophils out of 100 white blood cells then counted. This count was repeated 2 or 3 times and the mean ± SD is shown for HE values. HE = haematoxylin and eosin.

2. Flow cytometry compared to IHC method to assess percentages of CD4+ and CD8+ lymphocytes on human samples (n=5)

Following confirmation that the morphology of formalin fixed neutrophils and lymphocytes was adequately preserved to allow their identification on fixed blood clot sections, the next validation step was undertaken. This involved comparing results obtained by the conventional flow cytometry method to those obtained by the IHC method to measure percentages of CD4+ and CD8+ lymphocytes. This was performed on human blood since antibodies binding CD4 and CD8 for use in both fresh and fixed tissue are available for humans. The fresh blood samples were analysed using flow cytometry and the fixed blood clots were sectioned and stained for CD4 or CD8. Figure 3.1b, c shows representative staining of CD4+ and CD8+ lymphocytes.

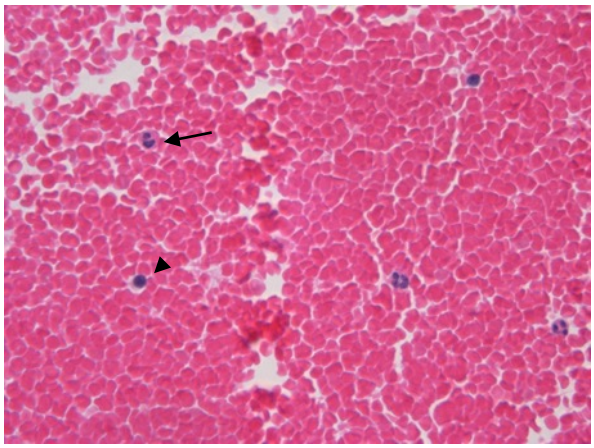
For the stained blood clot sections, the number of positive staining lymphocytes out of 100 total lymphocytes was recorded to provide the percentage of CD4+ or CD8+ lymphocytes. Student's paired t tests were performed to compare the results to those obtained by flow cytometry on the same blood samples. Although the IHC results for CD4 and CD8 percentages tended to be higher than the flow cytometry results, there was no overall significant difference between either the CD4+ ($p=0.062$) or CD8+ ($p=0.061$) percentages obtained by either method, or for CD4:CD8 ratio ($p = 0.510$) (Table 3.3).

Table 3.3. Comparison of CD4+ and CD8+ lymphocyte percentages obtained by two different methods for five individual humans.

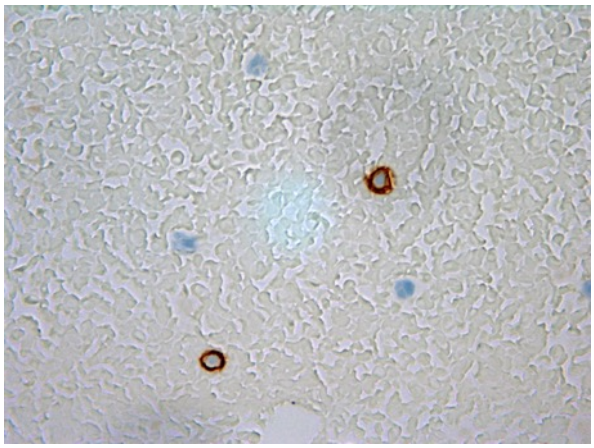
	CD4%		CD8%		CD4:CD8 ratio	
Human	Flow	IHC	Flow	IHC	Flow	IHC
A	40	47 ± 1	24	24 ± 2	1.7	2.0
B	62	65 ± 2	15	18 ± 2	4.1	3.6
C	43	43 ± 1	26	29 ± 1	1.7	1.5
D	53	55 ± 2	22	32 ± 1	2.4	1.7
E	55	57 ± 2	16	25 ± 2	3.4	2.2

The percentages of CD4+ and CD8+ lymphocytes for each of five blood samples were obtained by two different methods. Flow cytometry was performed by the hospital pathology laboratory which provided the results. Formalin fixed blood clots were stained with IHC with anti CD4 or CD8 antibodies, and the number of positive staining lymphocytes out of 100 lymphocytes then counted. This count was repeated 2 or 3 times and the mean ± SD is shown for IHC values. IHC = immunohistochemistry.

(a)



(b)



(c)

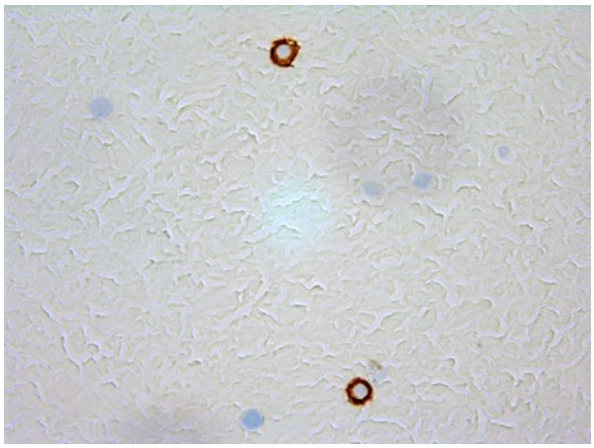


Figure 3.1. HE and IHC on blood clot sections, haematoxylin is the counterstain. (a) HE of devil blood clot showing 2 lymphocytes (e.g. arrow head) and 3 neutrophils (e.g. arrow). (b) CD4 of human blood clot section showing 2 positive (brown) staining lymphocytes, 3 negative staining lymphocytes (c) CD8 of human blood clot showing 2 positive (brown) staining lymphocytes, 5 negative staining lymphocytes. All images taken at 63 x objective.

3. Flow cytometry compared to IHC method to assess percentage of CD4+ lymphocytes on devil samples (n=4)

The final validation experiment was performed when a devil specific antibody binding CD4 became available toward the end of this study. The same steps that were performed on the human samples above were performed on blood samples from four devils to measure the percentage of CD4+ lymphocytes. Flow cytometry for CD4 was carried out on PBL's separated from the fresh (EDTA) blood samples for the four devils. Figure 3.2a shows the scatter plot and gating strategy for flow cytometry analysis of the PBL's. Figure 3.2b shows the clear separation of CD4+ and CD4- devil lymphocytes obtained by flow cytometry. Blood clots from the same four samples were fixed and sections stained with IHC for CD4 (Fig. 3.2c). The number of positive staining lymphocytes out of 100 total lymphocytes for each section was recorded to provide the percentage of CD4+ lymphocytes.

A Student's paired t test was performed to compare the results obtained by flow cytometry and IHC on the same blood samples. There was no significant difference for the overall results of CD4+ percentages obtained by either method ($p = 0.721$) (Table 3.4).

Table 3.4. Comparison of CD4+ lymphocyte percentages obtained by two different methods for four individual devils.

	CD4%	
devil	Flow	IHC
Batman	37	32 ± 2
Mars	23	30 ± 2
Prince	30	32 ± 1
Surprise	20	20 ± 2

The percentages of CD4+ lymphocytes for each of four blood samples were obtained by two different methods. Flow cytometry results are the rounded results obtained from Figure 3.2b. Formalin fixed blood clots were stained with IHC with anti-CD4 antibody and the number of positive staining cells out of 100 lymphocytes then counted. This count was repeated 2 or 3 times and the mean ± SD is shown for IHC values. IHC = immunohistochemistry.

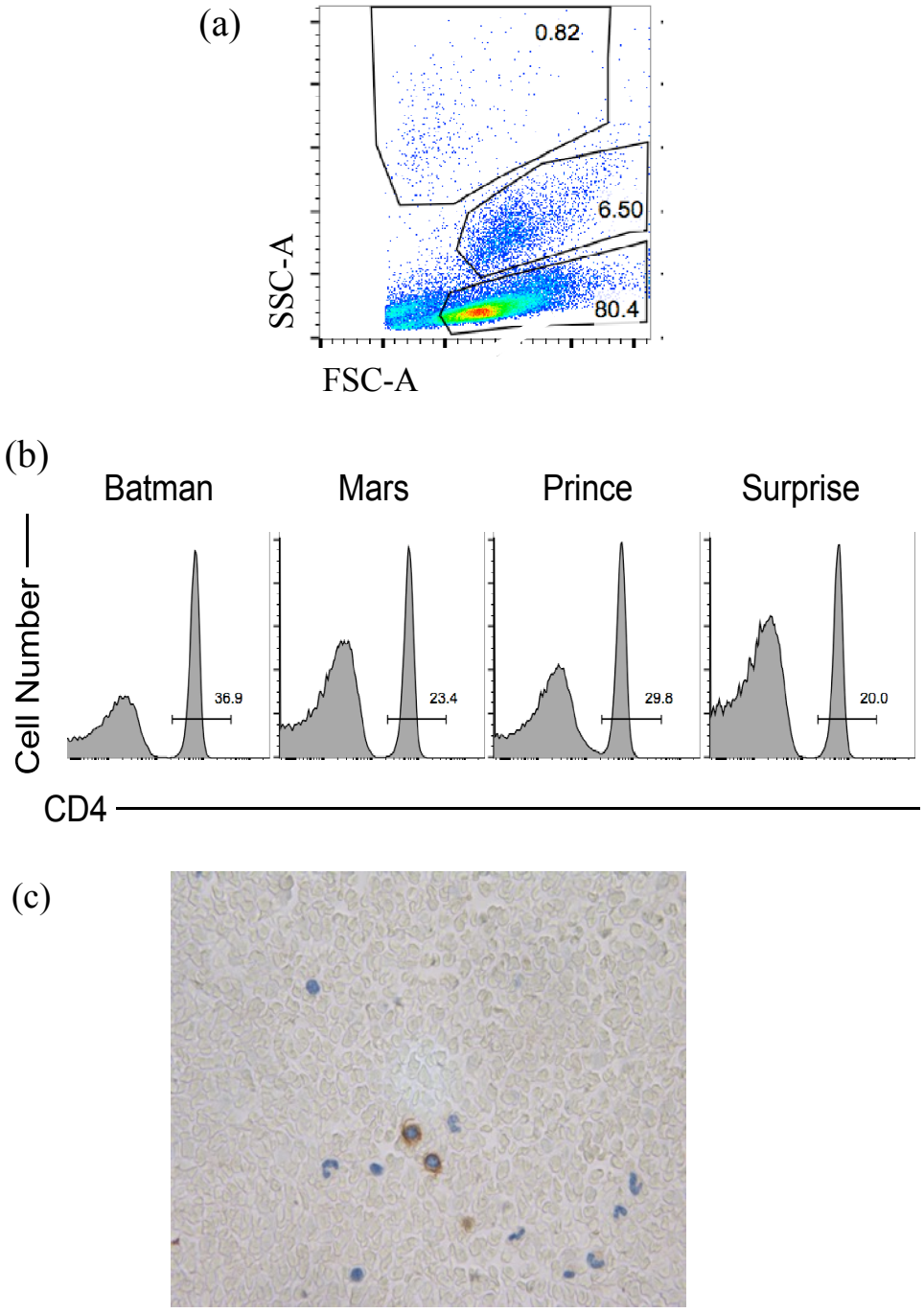


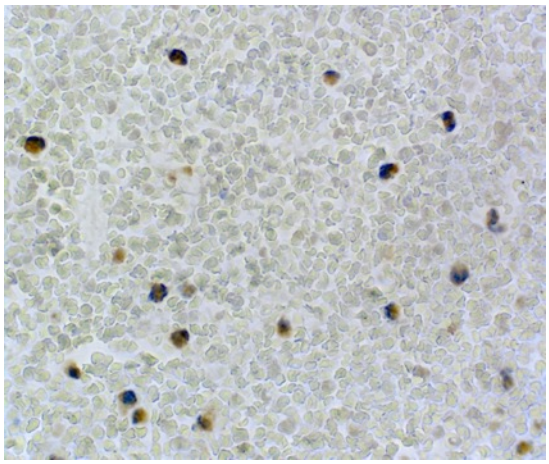
Figure 3.2. Peripheral blood lymphocyte (PBL) identification, and CD4 staining of fresh and fixed devil lymphocytes. (a) Flow plot of devil PBL's after isolation. The population closest to the x axis i.e. with 80.4% of cells, was identified as PBL's, (b) Flow cytometry histograms showing percentage of CD4+ lymphocytes of PBL's from four individual devils, (c) Immunohistochemistry of devil blood clot stained with anti-devil CD4 antibody (brown), haematoxylin is the counterstain. The image shows 2 CD4+ lymphocytes, 3 negative staining lymphocytes and 6 neutrophils. Image taken at 63 x objective.

3.3.3. CD79b and MHC-II IHC staining

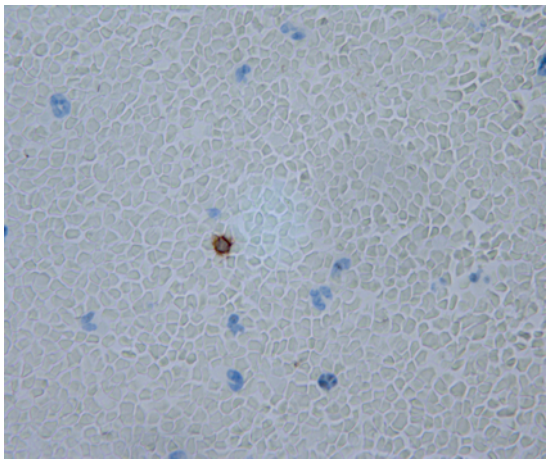
Cross-reactive staining of all white cells in the devil blood clots by the rat anti-mouse CD79b antibody precluded calculation of the percentages of B lymphocytes in the devils in this study (Fig. 3.3a). This is in contrast to the specific staining with anti-devil CD4 (Fig. 3.2c) and anti-devil CD8 (Fig. 3.3c). Consequently, 11 blood clot sections from devils with DFTD were stained with anti-MHC-II antibody. There appeared to be specific staining (Fig. 3.3b) but the number of positive cells was too low to quantify.

The validation experiments showed the IHC method to be a reliable way of measuring the percentages of CD4+ and CD8+ lymphocytes in the peripheral blood of devils. Although the values obtained by flow cytometry and IHC sometimes differed for individuals, the average values were not significantly different. It was this average that would be used to assess the percentages of lymphocytes in different devil cohorts.

(a)



(b)



(c)

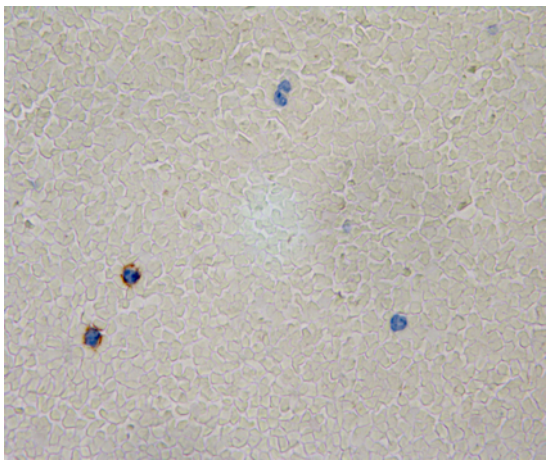


Figure 3.3. IHC of devil blood clots (positive cells are brown), haematoxylin is the counterstain (a) anti-mouse CD79b antibody: all cells have positive staining indicating the cross-reactivity of the CD79b antibody; (b) anti-human MHC-II antibody staining of a clot from a devil with DFTD: 1 MHC-II+ cell, 1 negative staining lymphocyte surrounded by neutrophils The large number of neutrophils is typical of the stress leukogram seen in devils with DFTD; (c) anti-devil CD8 antibody: 2 CD8+ lymphocytes, 1 negative staining lymphocyte, 1 granulocyte. Images taken at 63 x objective.

3.3.4. Percentages of CD4+ and CD8+ lymphocytes in peripheral blood of healthy devils. Effects of age, sex and season.

The percentages of CD4+ and CD8+ lymphocytes for each devil in this study were obtained by the novel method of formalin fixing clots from peripheral blood samples, and using IHC to stain them with anti-devil CD4 or CD8 antibodies. The percentage of positive staining lymphocytes out of 100 total lymphocytes was recorded. Percentages of CD4+ and CD8+ lymphocytes were initially evaluated for the 36 healthy devils (those without DFTD). The effects that age, sex and season had on these healthy devils were analysed with a three-way ANOVA.

Juvenile devils had a significantly higher percentage of CD4+ lymphocytes and lower percentage of CD8+ lymphocytes than adults, and consequently a significantly higher CD4:CD8 ratio than healthy adults (Fig. 3.4). There was no sex effect on percentages of CD4+ or CD8+ lymphocytes.

When the effects of season were analysed the only significant finding was noted for the CD4+ lymphocyte values. There was a significant increase in the percentage of CD4+ lymphocytes and the CD4:CD8 ratio in autumn (May) and winter (August) compared to spring (November) (Fig. 3.5). This seasonal analysis took into account the effects of age and sex. See Table 3.9 for detailed ANOVA results.

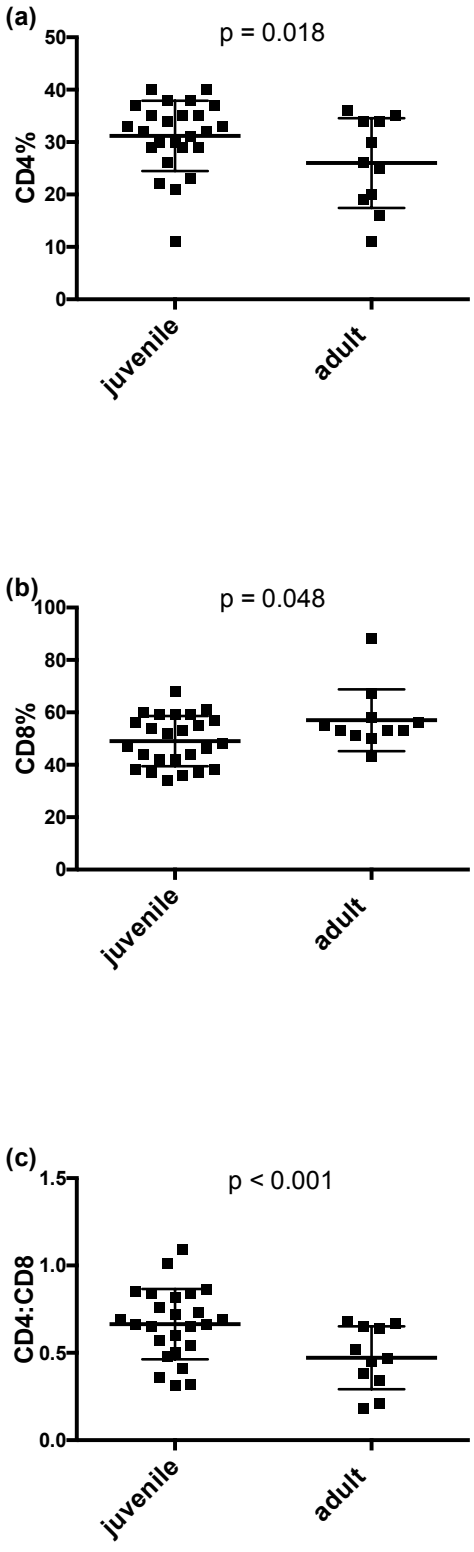


Figure 3.4. Effects of age on (a) CD4%, (b) CD8% and (c) CD4:CD8 ratio of healthy devils. Juvenile devils are < 2years old, adult devils are > 2years old. Statistical analysis was performed with a three-way ANOVA. The effects of sex and season are not shown on these graphs. Refer to Table 3.9 for detailed ANOVA results.

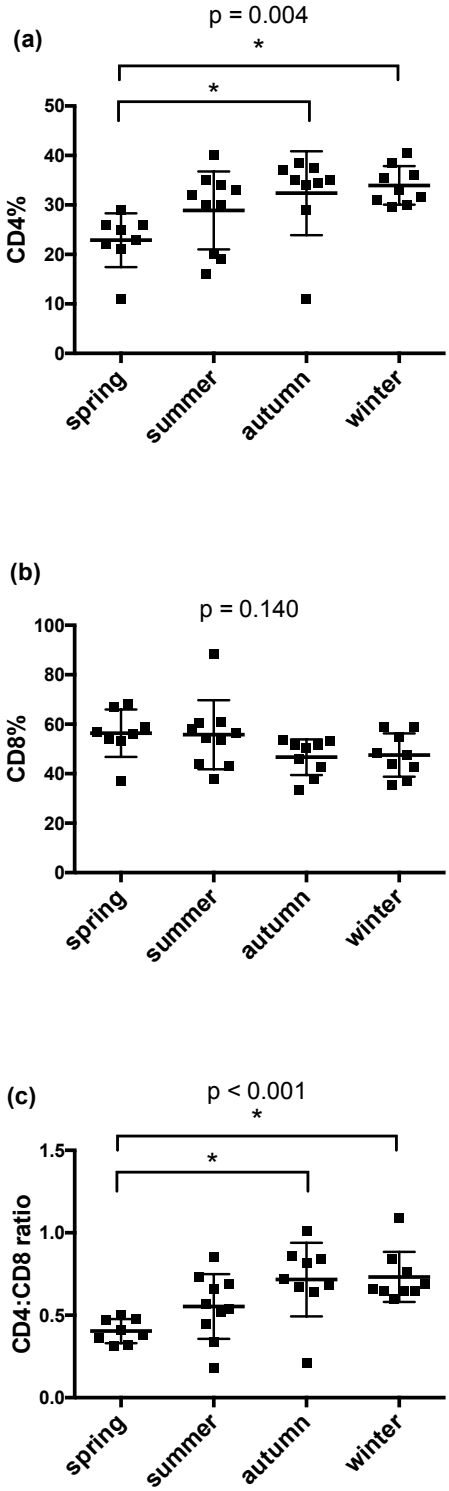


Figure 3.5. Effect of season on (a) CD4%, (b) CD8% and (c) CD4:CD8 ratio of healthy devils. Statistical analysis was performed with a three-way ANOVA to produce an overall p value for each graph. Post hoc analysis identified which seasons are significantly different from each other and are indicated by *. The effects of age and sex are not shown on these graphs. Refer to Table 3.9 for detailed ANOVA results.

3.3.5. Serum levels of IgM and IgG in peripheral blood of healthy devils. Effects of age, sex and season.

The serum levels of IgM and IgG were measured with ELISAs for each devil in the study. The values are recorded as optical density (OD) readings and are relative to each other. As with the CD4+ and CD8+ lymphocytes, IgM and IgG levels were initially evaluated for the 36 healthy devils, and the effects that age, sex and season had on these healthy devils were analysed with a three-way ANOVA.

The healthy juvenile devils had significantly lower IgM and IgG levels and a significantly higher IgM:IgG ratio than healthy adults (Fig. 3.6). As with the T lymphocyte subsets, sex had no effect.

There were no significant seasonal effects found on the immunoglobulin values (Fig. 3.7). As with the seasonal analysis of T lymphocyte subsets, the seasonal analysis of IgG and IgM also took into account the effects of age and sex. See Table 3.10 for detailed ANOVA results.

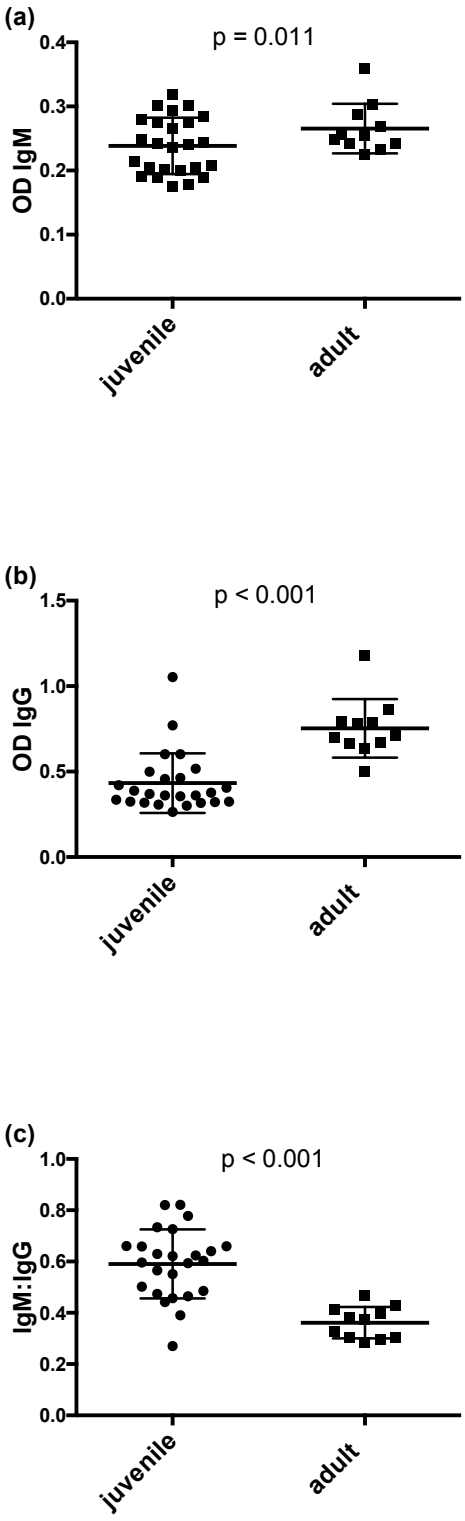


Figure 3.6. Effect of age on (a) IgM, (b) IgG and (c) IgM:IgG of healthy devils. Juvenile devils are < 2 years old, adult devils are > 2 years old. Statistical analysis was performed with a three-way ANOVA. The effects of sex and season are not shown on these graphs. Refer to Table 3.10 for detailed ANOVA results.

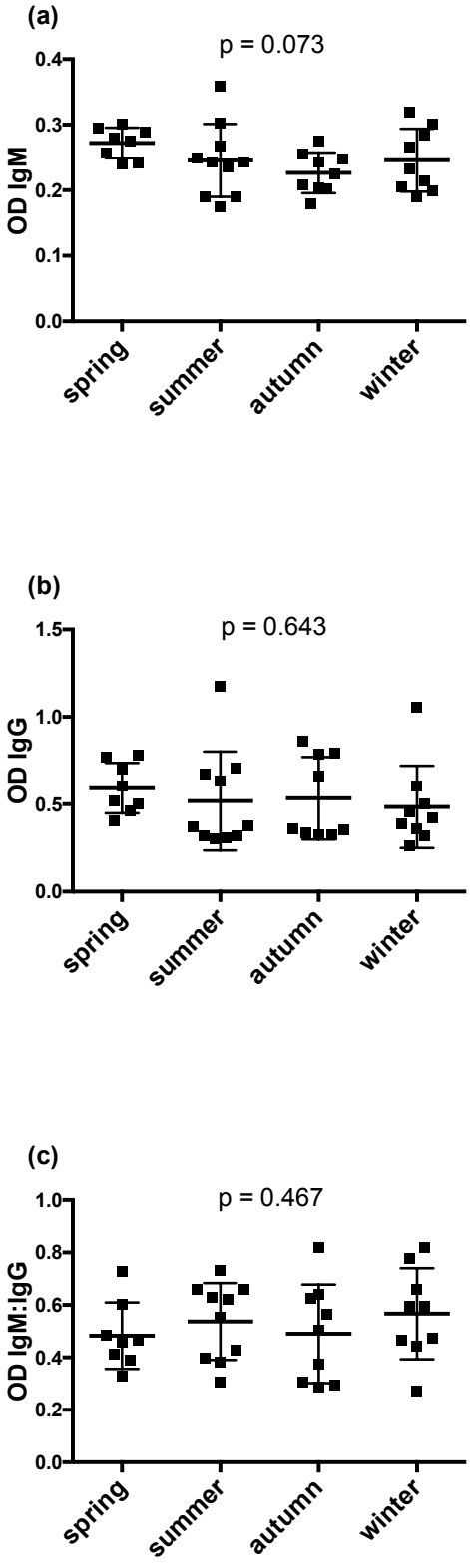


Figure 3.7. Effect of season on (a) IgM, (b) IgG and (c) IgM:IgG of healthy devils. Statistical analysis was performed with a three-way ANOVA to produce an overall p value for each graph. The effects of age and sex are not shown on these graphs. Refer to Table 3.10 for detailed ANOVA results.

3.3.6. The effect of DFTD on percentages of CD4+ and CD8+ lymphocytes in peripheral blood of devils

After determining percentages of CD4+ and CD8+ lymphocytes for healthy devils, these were then measured in devils with DFTD and the results compared. Values for healthy and diseased adult and juvenile devils are shown in Table 3.5. The table presents the average results from all devils tested and shows a decrease in CD4 and CD8 percentages for devils with DFTD.

Table 3.5. Percentages of CD4+ and CD8+ lymphocytes in peripheral blood of healthy and diseased devils.

	n		CD4%	CD8%	CD4:CD8 ratio
DFTD-	36		29.7 ± 7.7	51.6 ± 10.9	0.61 ± 0.21
DFTD+	22		14.8 ± 9.6	36.6 ± 17.1	0.41 ± 0.23
		n			
juvenile	DFTD-	25	31.3 ± 6.8	49.1 ± 9.6	0.66 ± 0.20
	DFTD+	6	14.0 ± 10.1	28.3 ± 13.0	0.50 ± 0.35
adult	DFTD-	11	26.1 ± 8.6	57.2 ±11.9	0.47 ± 0.18
	DFTD+	16	15.1 ± 9.7	39.7 ± 17.8	0.37 ± 0.16

Results represent mean percentage ± standard deviation for the number (n) of devils. DFTD- signifies healthy devils without DFTD; DFTD+ are devils with DFTD; juvenile devils are < 2 years old; adult devils are > 2 years old.

To analyse this further, data from individual devils were plotted and statistical analysis performed. As shown in Figure 3.8, devils with DFTD had significantly lower percentages of CD4+ and CD8+ lymphocytes, and lower CD4:CD8 ratios than healthy devils. The effect of both age and DFTD on CD4+ and CD8+ lymphocytes was then explored. As shown in Figure 3.9, when age was considered, the percentages of CD4+ and CD8+ lymphocytes were significantly higher in healthy devils than devils with DFTD for both age groups. However, despite DFTD reducing the CD4:CD8 ratio when all devils were included (Fig. 3.8c), this was not evident when age was considered (Fig. 3.9c). Taking age into account, DFTD was associated with a reduced percentage of both CD4+ and CD8+ lymphocytes for both age groups, but the CD4:CD8 ratio within each age group remained unchanged.

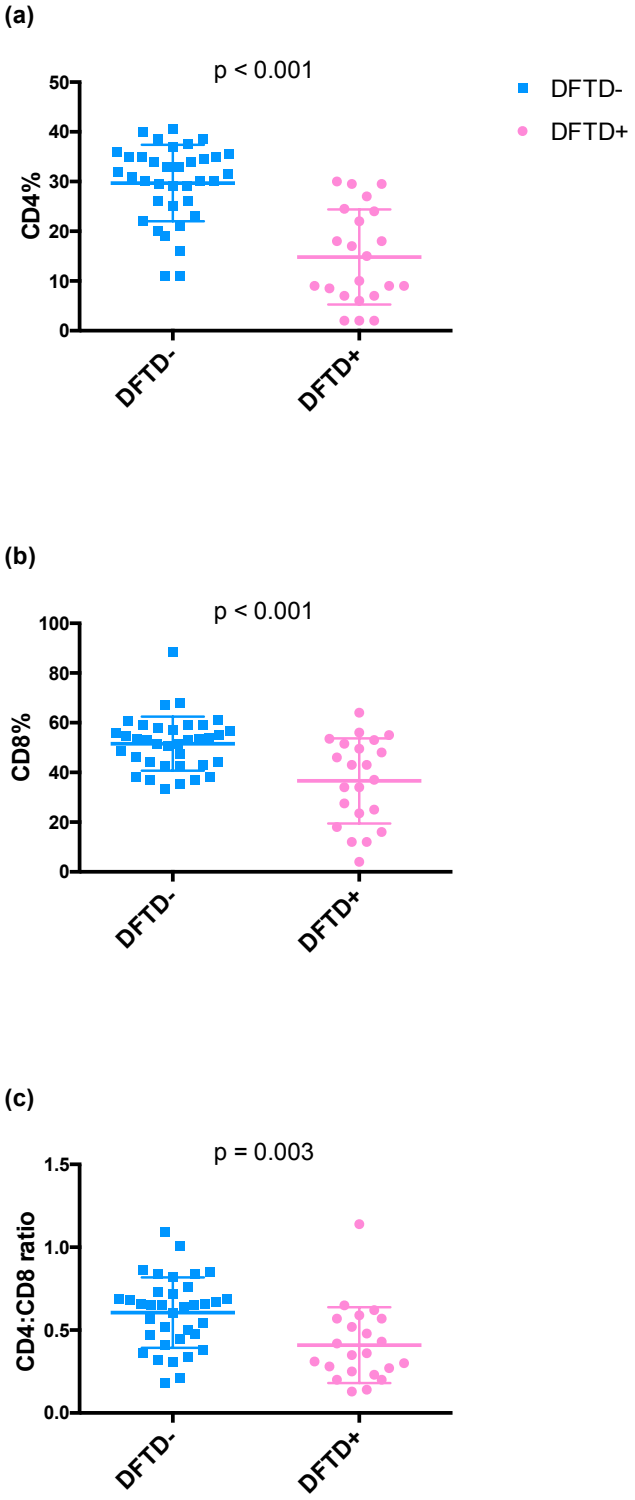


Figure 3.8. CD4+ and CD8+ lymphocytes in peripheral blood of healthy devils and devils with DFTD. (a) Percentage of CD4+ lymphocytes, (b) Percentage of CD8+ lymphocytes, and (c) CD4:CD8 ratio. Healthy devils (DFTD-) and devils with DFTD (DFTD+) were compared. Statistical analysis was performed using Student's unpaired t tests.

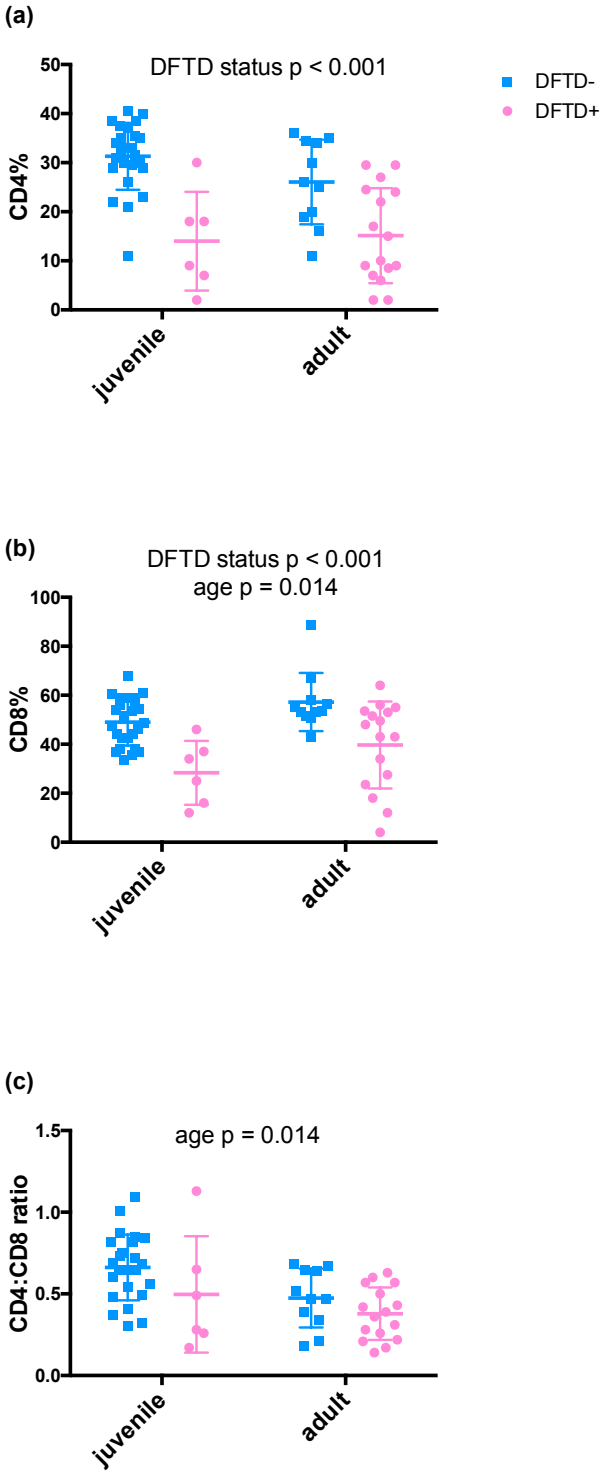


Figure 3.9. Percentages of (a) CD4+ lymphocytes, (b) CD8+ lymphocytes, and (c) CD4:CD8 ratio comparing healthy devils (DFTD-) and devils with DFTD (DFTD+) in juveniles and adults. Juvenile devils are < 2 years old, adult devils are > 2 years old. Statistical analysis was performed with a two-way ANOVA. Only significant p values are shown for age, DFTD status and/or interaction. Refer to Table 3. 12 for detailed ANOVA results.

As indicated above, devils with DFTD had lower percentages of CD4+ and CD8+ lymphocytes than healthy devils. There were fewer lymphocytes overall in the blood clots from devils with DFTD (approximately 9±3 per HPF in healthy devils compared to 5±3 per HPF in devils with DFTD). However, the proportion of non-staining lymphocytes was higher in devils with DFTD i.e. there were more lymphocytes that were CD4- CD8- in the clots from devils with DFTD (Table 3.6).

Table 3.6. Percentages of lymphocytes that are either CD4+, or CD8+, or CD4-CD8- in peripheral blood of healthy (DFTD-) and diseased (DFTD+) devils.

Lymphocytes	DFTD-	DFTD+
CD4+	29.7 ± 7.7	14.8 ± 9.6
CD8+	51.6 ± 10.9	36.6 ± 17.1
CD4+ or CD8+ (i.e. CD4% + CD8%)	81.3	51.4
both CD4- and CD8- (i.e. CD4-CD8-)	18.7	48.6

3.3.7. The effect of DFTD on serum levels of IgM and IgG in peripheral blood of devils

Analysis of total IgM and IgG was performed by an ELISA and the OD values were compared. Devils with DFTD had significantly higher IgM and IgG levels than healthy devils, whereas the IgM:IgG ratio was lower in diseased devils (Fig. 3.10).

Age influenced IgM and IgG levels in healthy devils (Fig. 3.6), so once again devils were divided into juvenile and adult cohorts. Age dictated whether a difference in IgM and IgG levels were found with diseased devils. Figure 3.11 summarises these results. For relative IgM levels, there was no significant difference in either age group (Fig. 3.11a). However, there was an interactive effect between age and DFTD status for IgG and the IgM:IgG ratio (Figs 3.11b,c). Healthy juvenile devils had lower relative IgG levels and a higher IgM:IgG ratio than juveniles with DFTD. There were no significant differences between the healthy and diseased adults. A summary of the effects of each parameter (age, sex, season and DFTD status) on the T lymphocyte subsets and IgM and IgG levels in peripheral blood is provided in Table 3.7.

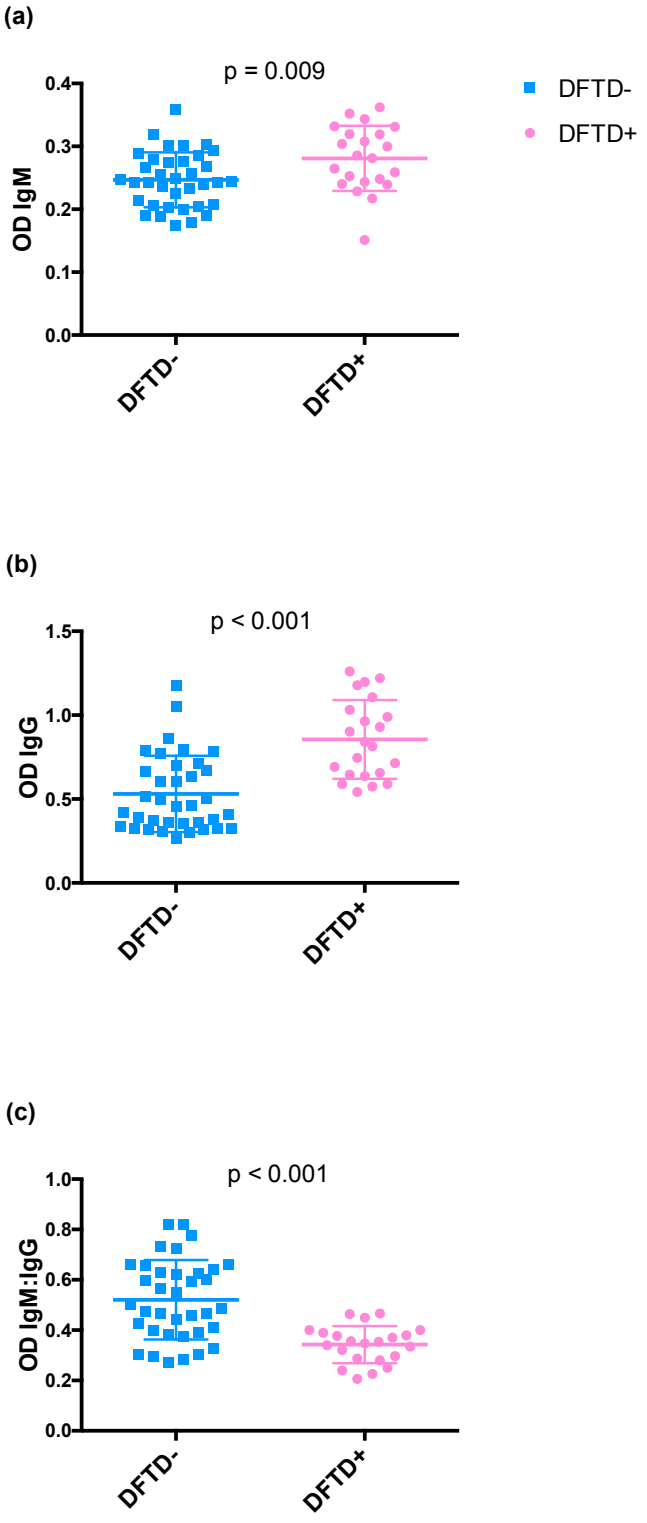


Figure 3.10. Serum levels of IgM and IgG in healthy devils and devils with DFTD. (a) Relative levels of IgM, (b) Relative levels of IgG, (c) IgM:IgG ratio. Healthy devils (DFTD-) and devils with DFTD (DFTD+) were compared. Statistical analysis was performed using Student's unpaired t tests.

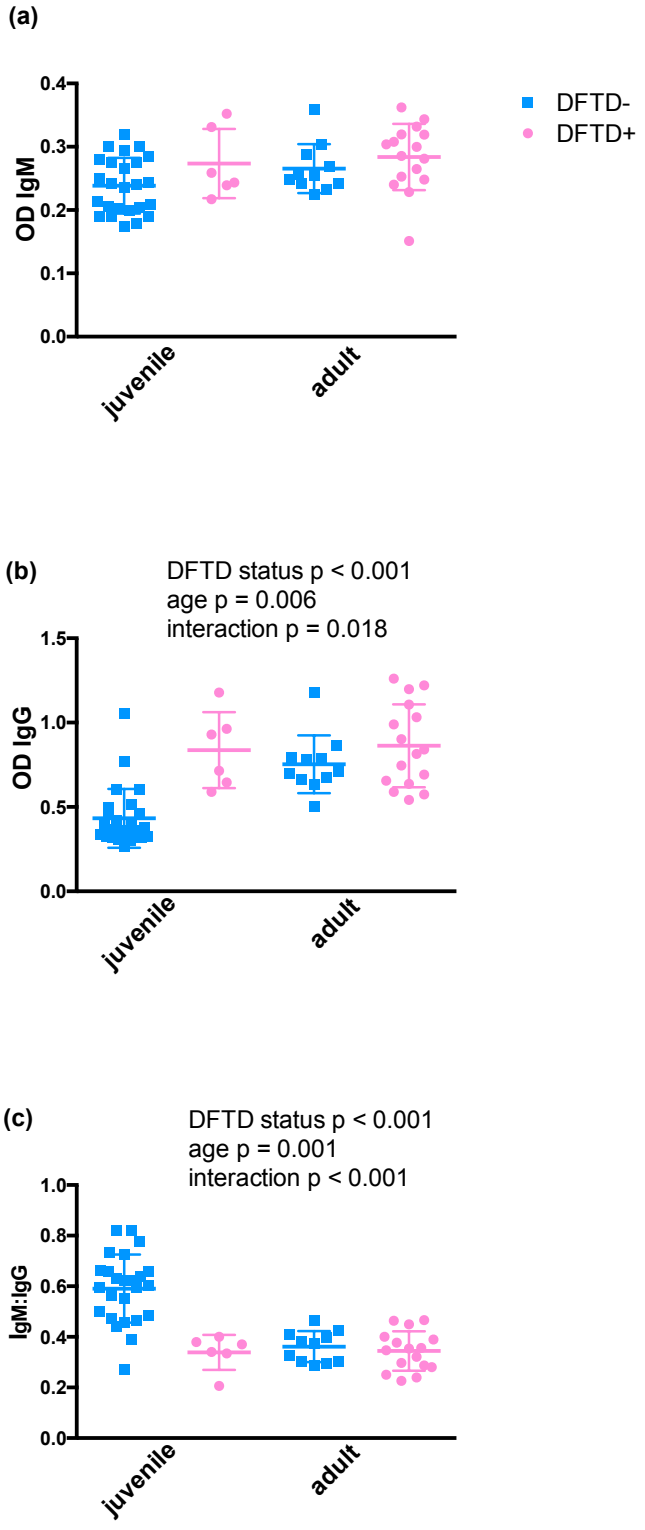


Figure 3.11. Serum levels of (a) IgM, (b) IgG, and (c) IgM:IgG ratio, comparing healthy devils (DFTD-) and devils with DFTD (DFTD+) in juveniles and adults. Juvenile devils are < 2 years old, adult devils are > 2 years old. Statistical analysis was performed with a two-way ANOVA. Only significant p values are shown for age, DFTD status and/or interaction. Refer to Table 3. 13 for detailed ANOVA results.

Table 3.7. Summary of the effects of age, sex, season and DFTD parameters on T lymphocyte subset populations and serum levels of IgM and IgG in peripheral blood of devils.

Devil cohorts compared for effects of each parameter	**CD4%**	**CD8%**	**CD4:CD8 ratio**	**IgM**	**IgG**	**IgM:IgG**
AGE						
Healthy juvenile devils (n=25) compared to healthy adult devils (n=11)	higher	lower	higher	lower	lower	higher
SEX						
Healthy male devils (n=17) compared to healthy female devils (n=19)	no difference	no difference	no difference	no difference	no difference	no difference
SEASON						
healthy devils spring n=8 summer n=10 autumn n=9 winter n=9	higher in autumn and winter compared to spring	no difference	higher in autumn and winter compared to spring	no difference	no difference	no difference
DFTD						
All devils with DFTD (n= 22) compared to all healthy devils (n=36)	lower	lower	lower	higher	higher	lower
Juvenile devils with DFTD (n=6) compared to healthy juvenile devils (n=25)	lower	lower	no difference	no difference	higher	lower
Adult devils with DFTD (n=16) compared to healthy adult devils (n=11)	lower	lower	no difference	no difference	no difference	no difference

n = number of devils

3.3.8. Statistical analysis

Table 3.8. The t and p values for Student's paired t tests (2 tailed) for the three different validation experiments.

	t	p
devil lymphocytes	0.147	0.890
devil neutrophils	1.812	0.144
human CD4	2.565	0.062
human CD8	2.592	0.061
human CD4:CD8	0.7228	0.510
devil CD4	0.393	0.721

Table 3.9. A three-way ANOVA for CD4, CD8 and CD4:CD8 ratio comparing effects of season, sex and age on healthy devils.

		CD4		CD8		CD4:CD8	
Parameter	Df	F	p	F	p	F	p
season	3	6.147	0.004**	2.034	0.14	11.955	<0.001***
sex	1	0.137	0.715	0.135	0.717	0.157	0.696
age	1	6.61	0.018*	4.406	0.048*	16.776	<0.001***
season:sex	3	1.295	0.302	1.091	0.375	3.793	0.026*
season:age	3	2.97	0.055	0.019*	0.996	1.958	0.151
sex:age	1	2.946	0.101	0.519	0.479	0.92	0.348
season:sex:age	2	0.312	0.736	0.349	0.709	0.342	0.714
residuals	21						

Significance codes: p < 0.001 '***'; p < 0.01 '**'; p< 0.05 '*'

Table 3.10. A three-way ANOVA for IgM, IgG and IgM:IgG comparing comparing effects of season, sex and age on healthy devils.

		IgM		IgG		IgM:IgG	
Parameter	Df	F	p	F	p	F	p
season	3	2.683	0.073	0.567	0.643	0.881	0.467
sex	1	3.151	0.090	1.86	0.187	0.217	0.646
age	1	7.731	0.011*	27.636	<0.001***	25.368	<0.001***
season:sex	3	2.823	0.064	2.823	0.064	1.43	0.262
season:age	3	1.911	0.159	1.911	0.159	0.579	0.635
sex:age	1	0.838	0.370	0.838	0.370	0.005	0.942
season:sex:age	2	2.794	0.084	2.794	0.084	0.307	0.739
residuals	21						

Significance codes: p < 0.001 '***'; p < 0.01 '**'; p< 0.05 '*'

Table 3.11. The t and p values for Student's unpaired t tests (2 tailed) for effect of DFTD on peripheral blood components i.e. comparing CD4, CD8, CD4:CD8 ratio, IgG, IgM, IgM:IgG in healthy devils and those with DFTD**.**

Peripheral blood components	t	p
CD4	6.510	<0.001 ***
CD8	3.906	<0.001***
CD4:CD8	3.109	0.003**
IgM	2.698	0.009**
IgG	5.216	<0.001 ***
IgM:IgG	4.960	<0.001 ***

Significance codes: p < 0.001 '***'; p < 0.01 '**'; p< 0.05 '*'

Table 3.12. A two-way ANOVA for CD4, CD8 and CD4:CD8 ratio comparing effects of DFTD and age.

		CD4		CD8		CD4:CD8	
	DF	F	p	F	p	F	p
Interaction	1	1.644	0.205	0.05875	0.809	0.2897	0.592
DFTD status	1	31.60	0.001***	22.51	<0.001***	3.614	0.063
age	1	0.7045	0.405	6.440	0.014*	6.419	0.014*
Residual	54						

Significance codes: p < 0.001 '***'; p < 0.01 '**'; p< 0.05 '*'

Table 3.13. A two-way ANOVA for IgM, IgG & IgM:IgG comparing effects of DFTD and age.

		IgM		IgG		IgM:IgG	
	DF	F	p	F	p	F	p
Interaction	1	0.3618	0.550	5.971	0.018*	14.12	<0.001***
DFTD status	1	3.648	0.063	18.14	<0.001***	18.64	<0.001***
age	1	1.766	0.190	8.251	0.006**	12.81	0.001**
Residual	54						

Significance codes: p < 0.001 '***'; p < 0.01 '**'; p< 0.05 '*'

3.4. Discussion

The T lymphocyte subsets and immunoglobulins of the peripheral blood provide an indication of health as well as insight into how cancer and other parameters influence the immune system. This study examined peripheral blood samples collected from a wild population of Tasmanian devils and found that DFTD was associated with reduced percentages of T lymphocyte subsets and, in juvenile devils, increased serum levels of IgG. It also found that season and/or age influenced T lymphocyte subsets and IgM and IgG serum levels in healthy devils.

For this study, a method using formalin fixed blood clots was developed. This overcame the difficulties of processing blood samples in the field and the limitations of available reagents. This method was used to analyse lymphocyte populations with routine HE and IHC staining. While a literature search revealed no previous use of formalin fixed blood clots for T lymphocyte subset analysis, they have been used for a variety of other purposes. These include identification of circulating canine neoplastic lymphocytes (Finlay and Wyatt, 2015), dengue viral antigen (Jessie et al., 2004), and babesia (Torres-Velez et al., 2003). Investigations of deep vein thrombosis and pulmonary thromboemboli in animal models (von Bruhl et al., 2012, Hand and Chandler, 1962) and sudden death syndrome (associated with blood clots in the heart) in chickens (Ononiwu et al., 1979) have also made use of formalin fixed blood clots.

The use and reliability of the formalin fixed blood clot method developed for this study was validated by three independent analyses. The first assessed whether cell morphology was adequately preserved in the clots to allow neutrophils and lymphocytes to be distinguished from each other and counted. The second analysis was performed on human blood samples since anti-human CD4 and CD8 antibodies are available for both fresh and fixed human tissue. This meant that results obtained by the conventional method for measuring percentages of T lymphocytes by flow cytometry could be compared to the IHC method. There was no overall significant difference between the results obtained by either method for the same samples. The final validation step was performed when, towards the end of this study, an anti-devil CD4 antibody became available for flow cytometry. The CD4+ T lymphocyte percentages in four devil blood samples were obtained by both flow cytometry and IHC staining and were not significantly different to each other.

The anti CD4+ antibody will bind to monocytes as well as lymphocytes. However, it was possible to distinguish monocytes in the sections by their lower levels of CD4 staining and their size and nuclear morphology. Monocytes could therefore be excluded from the manual count. In addition, since monocytes comprise only 0 - 2% of peripheral blood leucocytes for devils (Peck et al., 2015) and 2 – 10% in humans (Curry, 2015), they did not compromise the CD4+ T lymphocyte analysis.

All three analyses confirmed that the formalin fixed blood clot method was robust enough to evaluate peripheral blood T lymphocyte percentages of devil blood samples that were collected in the field. A particular reason for pursuing the method was to determine whether T lymphocytes were affected by DFTD. An alternative approach would have been to use blood smears. It can, however, be difficult to produce optimal blood smears in the field and there is a number of advantages the fixed sections have over smears. These include: no restriction to future analyses as occurs with the number of smears made at collection; stability of storage in paraffin blocks; fresh sections cut as required and/or as IHC reagents become available; formalin fixation and heat antigen retrieval can enhance staining; and, antibodies available for IHC may not be applicable to immunocytochemistry. The development of formalin fixed blood clots for lymphocyte analysis was a notable finding of this study.

The fixed blood clot method revealed that devils with DFTD had reduced percentages of CD4+ and CD8+ T lymphocytes compared to healthy devils. The absolute numbers of CD4+ and CD8+ T lymphocytes were not assessed in the present study since total white cell counts couldn't be performed in the field. However, devils with DFTD are known to have reduced total lymphocyte counts compared to healthy devils (Peck et al., 2016) and therefore the lower percentages of CD4+ and CD8+ T lymphocytes found in this study imply a reduced total number of T lymphocytes.

The lower percentages of peripheral T lymphocytes in devils with DFTD correlates with findings from a similar, but much larger (n=365) study on dogs with various cancers. Both percentages and absolute counts of lymphocyte subsets were measured (Watabe et al., 2011). The study explored how various cancers affected the peripheral blood lymphocyte subpopulations of dogs and by extension, their immune status. It found significantly lower percentages and absolute numbers of all lymphocyte phenotypes (CD4+ and CD8+ T

lymphocytes, and CD21+ B lymphocytes) in dogs with cancer. The conclusion was that advanced cancer caused marked immune compromise in these dogs.

The lower percentages of T lymphocyte subsets found in the blood of DFTD affected devils was associated with the higher percentage of non-staining (CD4-CD8-) lymphocytes. The absence of specific markers prevented identification of these cells. Potential candidates include B cells, NK cells, monocytes or NKT cells. The B lymphocyte population could not be calculated using the anti-mouse CD79b antibody as this was cross-reactive for all white cells in the blood clot sections. Clot sections from devils with DFTD were subsequently stained with anti-human MHC-II antibody to determine if the CD4-CD8- cells were B cells and/or antigen presenting cells. The MHC-II positive staining cells were too few to quantify implying the non-staining lymphocytes were not B cells. On a morphological basis, they were unlikely to be monocytes. NK cells and/or NKT cells are possibilities, although this seems unlikely given that NK cells represent only 8 to 10% of peripheral lymphocytes in humans (Whiteside, 2010). One possible explanation for the higher percentage of CD4-CD8- lymphocytes in devils with DFTD is provided by Watabe et al whose study found a similar population of non-staining lymphocytes in dogs with cancer compared to healthy controls (Watabe et al., 2011). The authors suggested that immunosuppressive cytokines secreted by tumours might down-regulate expression of CD3 and CD21 molecules. A similar process has been demonstrated in people with cancer whereby the density of CD antigen expression (CD3, CD4, CD8 and CD28) on lymphocyte populations was lower when compared to healthy donors (Hellstrom et al., 2001). Similarly, tuberculosis patients have shown an increased proportion of double negative staining T cells (Pinheiro et al., 2012). The lower percentages of CD4+ and CD8+ lymphocytes and corresponding increase in non-staining lymphocytes found in devils with DFTD might therefore be explained by DFTD-associated cytokines down-regulating expression of CD4 and CD8 on lymphocyte populations. The increased percentage of CD4-CD8- lymphocytes was an unexpected finding of this study and although there are plausible explanations, the phenotype of these cells requires further investigation.

Another unanticipated result of this study was the lower proportion of CD4+ than CD8+ lymphocytes found in the peripheral blood of devils. This occurred across all parameters of DFTD status, age, sex and season and is the inverse of the usual CD4/CD8 proportions found in most eutherian mammals (see Fig. 3.12).

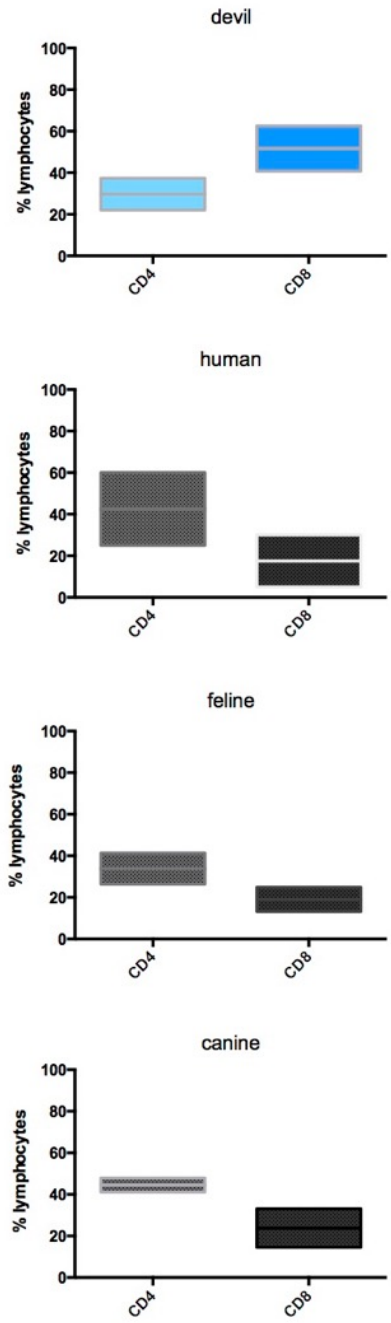


Figure 3.12. Proportion of percentages of CD4+ and CD8+ T lymphocytes found in devils and three eutherian mammal species. (Chng et al., 2004, Dean et al., 1991, Itoh et al., 2009).

Humans and most domestic eutherian mammals whose lymphocyte subset populations have been calculated e.g. cats, dogs, cattle and horses, have a higher proportion of CD4+ than CD8+ T lymphocytes in the peripheral blood and lymphoid organs. This same proportion occurs in devil spleen and lymph nodes (Howson et al., 2014). Koalas are the only other marsupial species to have their peripheral blood lymphocyte subsets examined and, in common with the devils, they have a higher percentage of CD8+ than CD4+ lymphocytes in their peripheral

blood (Chandan Mangar pers. comms, August 2016). CD4+ lymphocytes represent 23.8% (range 17.3 – 35%) of koala peripheral blood lymphocytes (Mangar et al., 2016) which is similar to the 29.7 ± 7.7% identified in devil peripheral blood. It is notable that domestic pigs share this same inverse proportion of CD4+ and CD8+ lymphocytes (Saalmuller et al., 1999).

There are at least two differences between eutherian mammals and marsupials regarding their T lymphocytes which have been documented and may partially explain the inverse proportion identified in devils and koalas. The first relates to regulatory T cells (Tregs), a subset of T lymphocytes that dampen the immune response and have a role in preventing autoimmune disease (Jiang et al., 2014). Tregs arise in the thymus (tTregs) and in the periphery (pTregs). They are CD4 positive thus contributing to the total CD4+ T cell count. The conserved noncoding sequence 1 (CNS1) is essential for pTreg cell generation, and CNS1 is present in eutherian mammals but absent in monotremes and marsupials (Samstein et al., 2012). Eutherian mammals require pTregs to protect against maternal-foetal conflict and allow for prolonged *in utero* foetal development. Indeed, abortion is associated with women who have a pTreg deficiency (Sasaki et al., 2004). Marsupial young spend only a brief period of time in the placenta (21 days for devils) and undergo the majority of their development in the maternal pouch. Although there are no reagents available to identify and confirm the absence of pTregs in the peripheral blood of marsupials, the short gestation period and the genetic evidence described above suggest they are not present. The lack of CD4+ Tregs in the peripheral blood of devils might provide some explanation for the reduced percentage of CD4+ T lymphocytes. However, Tregs in the peripheral blood of humans account for 5 to 10% of total CD4+ cells (Boye et al., 2010) and thus the lack of pTregs doesn't entirely explain the inverse proportion.

A second difference found in the marsupial T cell population is the existence of a unique T cell receptor, TCRμ (μ for marsupial) (Parra et al., 2007). It is uncertain whether these cells are CD4 or CD8 positive, however there is evidence they are co-expressing the γ chain i.e. are γμ T cells. The μ chain shares an ancestry with δ and these cells may therefore have characteristics of the γδ cell phenotype (Rob Miller pers. comms, February 2016). In humans CD4 and CD8 molecules are uncommon on γδ T cells (Deniger et al., 2014) and therefore cells expressing TCRμ might be negative for CD4 and/or CD8. It seems too speculative at this stage to consider if these cells account for the CD4-CD8- cells associated with DFTD that were identified in this study. While these differences don't provide a direct explanation for the inverse proportion of

CD4/CD8 lymphocytes found in devils, it can be said they exemplify that differences between species, and between marsupials and eutherians, exist.

Of course, many similarities are found between the immune systems of eutherian and marsupial species and this was true when assessing the effect age had on T lymphocyte subsets. Juvenile devils had higher mean CD4+ and lower mean CD8+ percentages than adults. These findings correlate with various studies on dogs and cats that have addressed age-associated changes to the immune system. A decrease in the percentage of CD4+ T lymphocytes, and an increase in that of CD8+ T lymphocytes was associated with increasing age in healthy dogs across a number of studies (Watabe et al., 2011, HogenEsch et al., 2004, Heaton et al., 2002a, Greeley et al., 1996). Similar results have been found in cats (Heaton et al., 2002b). The first report of age related variation in immunity in a wild mammal population was on a group of Soay sheep where T lymphocyte subsets were measured (Nussey et al., 2012). While the CD8+ lymphocyte proportion increased with age, the proportion of CD4+ lymphocytes in Soay sheep also increased (although naïve CD4 cells (CD45RA+) decreased).

The age-associated percentage increase of CD8+ lymphocytes found across species, including devils, might be explained by the T cell clonal expansions (TCEs) that occur with aging. Large TCEs are almost always CD8+, are most strongly associated with age, and impact on immune responsiveness due to loss of T cell receptor diversity. As such, the CD8+ TCE's are believed to contribute to the "immunodeficiency of senescence" (Messaoudi et al., 2004). Likewise, the reduced population of naïve T lymphocytes and increase in memory T cells explain the age associated decrease in CD4+ and CD8+ mediated responses (Linton and Dorshkind, 2004).

Seasonal effects on immune function are most commonly explained by adrenal corticosteroids which dampen immune function and T cell activity. Their increased secretion in winter and decrease in summer link them to the circannual change in immune function (Fares, 2013). This study found a significant seasonal effect on the percentage of CD4+ T lymphocytes in healthy devils with an increase in autumn/ winter compared to spring/ summer. These (CD4+) findings correlate with at least two human studies (Termorshuizen et al., 2002, Paglieroni and Holland, 1994) and tie in with seasonal changes found in adult male devils whereby stress leukograms (reduced total lymphocyte and increased total neutrophil counts) and reduced body weights occurred in the post-mating season of autumn (Peck et al., 2015).

Tasmanian devils with DFTD had higher levels of both IgM and IgG which correlates with results from a previous study that showed devils with DFTD had higher globulin levels than healthy devils (Peck et al., 2016). Electrophoresis results for serum samples from 12 devils showed the gamma globulin proportion to be similar for healthy and diseased devils (Appendix 1). The alpha 1 globulin component was notably higher in devils with DFTD, and there was a tendency for the beta 1 and beta 2 fractions to be elevated in devils with DFTD when compared to healthy devils. IgA, IgM and sometimes IgG can be found in the beta fraction (O'Connell et al., 2005). It was reported by Peck et al that when age was considered alongside DFTD, the globulin levels for adults with DFTD and healthy adults were the same (Peck et al., 2016). Likewise, when age was factored in to the assessment of DFTD's effect on IgM and IgG levels, the increased IgG levels associated with DFTD were only found in juvenile devils. There was no effect on IgM. It therefore appears that the major effect of DFTD with respect to the immunoglobulins analysed is restricted to increasing IgG levels in juvenile devils and a corresponding reduction in their IgM:IgG ratio. A large proportion of DFTs are ulcerated (74%) and/or necrotic (73%) (Loh et al., 2006) and this may allow for bacterial infection of the tumours (Peck et al., 2016). Increased pathogen exposure associated with DFTD might therefore account for the increased globulins. These results contrasted somewhat to those of a recent study which found DFTD was associated with a lower IgM:IgG ratio, independent of age (Ujvari et al., 2016). The authors suggested the IgM:IgG ratio in devils may play a role in devil susceptibility to DFTD. That study had a smaller sample size (n=23) than the one described in this thesis, and used mRNA rather than protein for analysis. A longitudinal study would allow further exploration of the IgM:IgG association with DFTD.

With respect to healthy devils, serum levels of IgM and in particular IgG were significantly increased in adult devils. These findings correlate with a previous report showing that the serum globulin range for healthy adult devils (29-46 g/L) was higher than the range for healthy juveniles (22-41 g/L) (Peck et al., 2015). The same explanation suggested above might account for these differences i.e. cumulative antibody responses to increased pathogen exposure which occurs over time. Humans also show increasing IgM and IgG levels with age (up to middle age) (Jazayeri et al., 2013, Gonzalez-Quintela et al., 2008). Similarly, a study comparing IgM and IgG serum levels in captive and wild hyenas found significantly higher serum levels of both in wild hyenas (Flies et al., 2015). Although the study didn't compare hyena ages, the increased exposure to pathogens experienced by the wild cohort was the explanation proposed for the differences. This ties in with the devil findings in so far as increased exposure occurs

with age. In addition, it is expected the naïve juvenile immune systems would have relatively less IgG since IgG is class switching i.e. is made in response to antigen stimulation and thus increases with exposure to insults and infection as occurs with aging.

In conclusion, this study showed that DFTD altered some of the immune components of the peripheral blood of devils. The most consistent finding in both adult and juvenile devils with DFTD was a reduction in the CD4+ and CD8+ lymphocyte populations. This could interfere with cell mediated immune responses and allow the progression of DFTD. The effect of DFTD on immunoglobulin levels was restricted to juvenile devils which had an increase in IgG levels. This might be explained by increased pathogen exposure secondary to DFTD rather than a direct result of DFTD. This study also showed how devils' peripheral blood lymphocytes and immunoglobulins compared to those of other species. These were influenced in a similar fashion by the parameters of age and season. One notable difference however was the proportion of CD4/CD8 lymphocytes in devils which was the inverse of most eutherian mammals. Although similarities across species clearly exist, demonstrated differences should not be dismissed. Such differences may have consequences for the mechanisms which regulate the development and expression of immune responses to disease, including cancer and should be considered when exploring the interplay between DFTD and the devil's immune system.

Chapter 4

Demonstration of immune responses against devil facial tumour disease in wild Tasmanian devils

Chapter 4. Demonstration of immune responses against devil facial tumour disease in wild Tasmanian devils

4.1. Introduction

Devil facial tumour disease (DFTD) is a recently emerged transmissible cancer threatening the Tasmanian devil with extinction in the wild (Hawkins et al., 2008). It is transmitted when susceptible and infected individuals bite each other and is considered invariably fatal, with most animals dying within 6 to12 months of the tumour first appearing (Hamede et al., 2012). The literature reports that no protective immune response has been observed (Siddle and Kaufman, 2015).

The disease is transmitted as an allograft (Pearse and Swift, 2006) and three explanations were initially suggested to explain the lack of immune rejection: the limited genetic diversity of the species; the unknown competency of the devil's immune system; and the immune evasion mechanisms of the tumour (Woods et al., 2015). All research to date addressing these possibilities suggests it is the successful immune evasion strategies employed by the tumour cells that are primarily responsible for the transmission of DFTD (Woods et al., 2015, Siddle and Kaufman, 2013, Siddle et al., 2013).

Current DFTD research suggests that a major mechanism of immune escape is down-regulation of the major histocompatibility complex class I molecule (MHC-I) (Siddle et al., 2013). MHC-I cell surface expression occurs on all nucleated cells and allows the immune system to recognise foreign or infected cells. Some cancers fail to express surface MHC-I, a mechanism which contributes to evasion of the host's T cell response. The only other naturally occurring transmissible cancer to affect a mammalian species is canine transmissible venereal tumour (CTVT) in domestic dogs. CTVT also demonstrates MHC-I down-regulation in its progressive phase (Hsiao et al., 2002). However, after three to four months of tumour growth, there is increased surface MHC-I expression resulting in a host alloresponse. This is demonstrated by host antibody production and T lymphocyte infiltration of the tumour resulting in tumour stabilisation or regression and immunological memory. MHC-I expression of CTVT is associated with the presence of inflammatory cytokines (Hsiao et al., 2002, Cohen, 1972). Likewise DFTD's down-regulation of surface MHC-I can be reversed *in vitro* by treatment of DFTD cells with the inflammatory cytokine interferon gamma (IFN-γ) (Siddle et al., 2013).

Down-regulation of MHC-I provides an explanation for DFTD transmission and is believed to be responsible for the lack of a T cell mediated immune response against the tumour. The long-standing assumptions are that DFTD always escapes the devils' immune system, and that the disease is invariably fatal. These assumptions were re-examined in this study by analysing serum and tumour samples from a population of wild devils to detect the presence of anti-DFTD immune responses.

4.2. Materials and methods

4.2.1. Individual devils and sample collection

The samples used in this study were collected between 2008 and 2014 from a closely monitored population at West Pencil Pine in north western Tasmania. Blood samples were collected from a total of 52 devils. Ages ranged from one to six years, with 34 females and 18 males (Table 4.1). Tumour samples were collected from a total of 20 devils. Tumour sample collection was dependent on size and location of the tumour, and was not always possible in a field setting. Tumour size was measured by recording length, width and depth with 15 cm caliMax calipers. See Chapter 2.3.3 and 2.3.4 for blood and biopsy collection, and 2.3.5 for fine needle aspirate collection for subsequent cytology, karyotyping or immunocytochemistry.

Table 4.1. Summary of serum samples collected and analysed from Tasmanian devils from West Pencil Pine.

Age (years)	**1**	**2**	**3**	**4**	**5**	**6**	**Total**
Number of devils	16	15	11	4	4	2	52
DFTD+ (including those with regressed tumours)	5	10	11	3	3	2	34
DFTD-	11	5	0	1	1	0	18
Females	8	8	10	3	4	1	34
Males	8	7	1	1	0	1	18
Females DFTD+	2	6	10	2	3	1	24
Males DFTD+	3	4	1	1	0	1	10
Number of devils that had only 1 serum sample analysed	5	2	0	0	0	0	7
Number of devils that had multiple serum samples analysed	11	13	11	4	4	2	45
Number of devils that had serum taken prior and subsequent to becoming DFTD+ (including those with regressed tumours)	0	4	7	2	2	2	17
Number of devils that had their final serum sample taken at time of first clinical signs	3	6	4	1	0	0	14
Number of DFTD- devils that had multiple serum samples analysed	8	4	0	1	1	0	14

Blood was collected from a further 20 devils from a DFTD free island to use as the negative control serum (Table 4.2). The serum samples had been collected > 6 months after release of the devils on to the island. The adult (> 1 year old) females had higher levels of background IgG antibody than the other cohorts so their sera were used as the negative control for the wild devils from West Pencil Pine.

Table 4.2. Summary of samples collected and analysed from Tasmanian devils from Maria Island (DFTD free island) used as the negative control serum.

	1 year old	Adult (> 1 year old)
Males	5	5
Females	5	5

4.2.2. DFTD assessment

In cases where histopathological samples were not taken, fine needle aspirate (FNA) collection and either cytological examination (one devil), cell culture and karyotype (six devils), or immunocytochemistry (ICC) (five devils, two of which also had biopsies collected) were taken from cutaneous or oral raised nodular or ulcerated lesions. The results of these methods supported DFTD identification. For the remaining 14 devils, visual assessment of tumour location and appearance according to previously described classification methods (Hawkins et al., 2006) supported DFTD identification. Table 4.3 summarises the techniques used to identify DFTD for each devil in this study. See Chapter 2.2.10 for karyotype method.

Table 4.3. Summary of techniques used to identify DFTD in the 34 DFTD+ devils.

Diagnostic technique	No. of DFTD cases	Veterinary pathologist report/ periaxin positive
Visual diagnosis	14	/
Histopathology (biopsy)	11 (including TD3's DFT1 recurrence)	11
Cytology: May Grunwald-Giemsa stain	1	1
Cytology: immunocytochemistry	5 (2 of these also had biopsies from which a histology diagnosis was made)	5
Cell culture & karyotype	6	6

"/" indicates not performed

Histopathology and immunohistochemistry interpretation

A diagnosis of DFTD was made based on clinical findings supported by histopathological examination of tumour biopsies for ten devils. Histopathology was assessed by a veterinary pathologist. Confirmation of DFTD by HE stained sections was based on histopathologic findings consistent with Loh et al (Loh et al., 2006). Immunohistochemistry (CD3 and MHC-II) was performed on the nine tumour biopsies available for this study. See Chapter 2.2.1 for the IHC staining procedure. Assessment of tumour infiltrating lymphocytes in anti-CD3 antibody stained sections was performed by a veterinary pathologist and based on the method outlined by Zhang et al (Zhang et al., 2003) and reviewed by Gooden et al (Gooden et al., 2011). A similar method for counting the number of infiltrating positive MHC-II cells was performed.

Immunocytochemistry

Immunocytochemistry (ICC) was performed by Alison Caldwell, Southampton on five tumour fine needle aspirates (FNA's). The samples were stained for periaxin, a positive marker for DFTD cells (Tovar et al., 2011), and for $\beta_2$microglobulin (β_2m), a component of the MHC-I molecule.

From each FNA sample, 100 µl was seeded onto poly-L-lysine (Sigma P4707) coated slides. Slides were incubated for 60 minutes at room temperature. Excess cell suspension was aspirated and slides placed in PBS for 10 minutes. Cells were incubated in ice-cold methanol for 10 minutes and air-dried, before incubation in blocking buffer (10 % goat sera in PBS) for 30 minutes to minimize any non-specific absorption of antibodies. Cells were incubated in anti-devil β_2m antibody at 20 µg/ml and periaxin in blocking buffer for 30 minutes. Cells were washed twice in PBS for 10 minutes and then 5 minutes. The secondary antibodies (goat anti-mouse Alexa Fluor® 488 and goat anti-rabbit Alexa Fluor® 647) were diluted 1:1000 in PBS and cells were incubated for 30 minutes. Cells were washed three times in PBS for 5 minutes. Slides were mounted with DAPI mounting media (Fluoroshield™ with DAPI, Sigma).

Serum antibody detection

Indirect immunofluorescence and flow cytometry to measure serum anti-DFTD IgG antibody levels was performed on the serum samples against un-manipulated DFTD cells (i.e. cells not expressing MHC-I, referred to as MHC-I^{-ve} DFTD cells); and cells treated with IFN-γ to up-regulate MHC-I expression i.e. MHC-I^{+ve} DFTD cells. For 45 of the individual devils, multiple serum samples collected over an extended period were analysed. Sera from a translocated population of captive born devils living in wild conditions on a DFTD-free island were used as the negative control. See Chapter 2 for MHC-I up-regulation (2.2.5) and flow cytometry (2.2.7) procedures. Antibody levels were recorded as median fluorescence intensity (MFI).

4.3. Results

This study aimed to identify an immune response against DFTD by examining serum and tumour samples from a population of wild devils. Of the 52 devils, 46 had no detectable serum IgG antibody against either MHC-I^{+ve} or MHC-I^{-ve} DFTD cells. The remaining six devils (referred to here as TD1 through to TD6) had serum IgG antibody against MHC-I^{+ve} DFTD cells, but not MHC-I^{-ve} cells. Only one of these six devils had clinical signs of DFTD at initial sample collection. The remaining five devils developed DFTD at some stage during sample collection (Table 4.4). DFTD assessment was made in TD1, TD2 and TD3 by visual examination; in TD4 by cytology of the FNA; in TD5 by histopathology; and in TD6 by ICC of the FNAs.

Multiple serum samples from each of the six devils were analysed and for each devil the earliest sample had the same MFI as the negative control. After these devils showed clinical signs of DFTD, they developed anti-DFTD antibodies (Fig. 4.1). Remarkably, DFTD regression occurred in four of the six devils that had seroconverted (TD1, TD2, TD3 and TD4). When each devil was retrapped between 4 and 15 months after DFTD was first noted, their tumours were no longer visible and anti-DFTD antibodies were detected. TD1 and TD2 were not retrapped more than three months after the regression was observed. TD3 remained disease-free for two years following tumour regression but at the age of five years, a tumour biopsy confirmed recurrence of DFTD. Serum antibodies persisted in TD3 at this time and tumour-infiltrating MHC-II+ cells and CD3+ T lymphocytes were present in the biopsy (Table 4.5). TD4 remained disease free for three years to the age of six years, beyond which it was not retrapped (six years is considered the maximum lifespan for a wild devil).

Table 4.4. DFTD and antibody (Ab) status of 6 Tasmanian devils exhibiting anti-DFTD responses

Devil ID.	YOB	sex	date	DFTD status	serum Ab's	Comments
TD1	2006	F	Nov 08- May 11 Jul-11 Nov-11 Feb-12	D- D+ D- D-	negative / medium medium	 tumour regressed (not retrapped after Feb 12)
TD2	2006	F	Feb 09- Feb 11 May-11 Nov-11	D- D+ D-	negative medium high	 tumour regressed (not retrapped after Nov 11)
TD3	2009	F	Feb-11 Feb-12 May-12 Feb-14 May-14	D- D+ D- D- D+	negative negative / high high	 tumour regressed disease free for 2 years DFTD, T lymphocytes in biopsy (not retrapped after May 14)
TD4	2008	M	Jul-11 Aug-12 Nov-13 May-14	D- D+ D- D-	negative / medium high	 tumour regressed still disease free (not retrapped after Aug 15)
TD5	2008	F	Feb 09-Nov 10 Feb-11 May-11	D- D+ D+	negative negative high	 T lymphocytes in biopsy (not retrapped after May 11)
TD6	2010	M	Nov-13 Feb-14	D+ D+	negative high	 MHC^{+ve} DFTD cells in FNA (not retrapped after Feb 14)

Serum Ab column: "/" no serum sample collected, "negative" same as MFI control, "medium" 2 - 4 x MFI control, "high" > 4 x MFI control)

YOB = year of birth

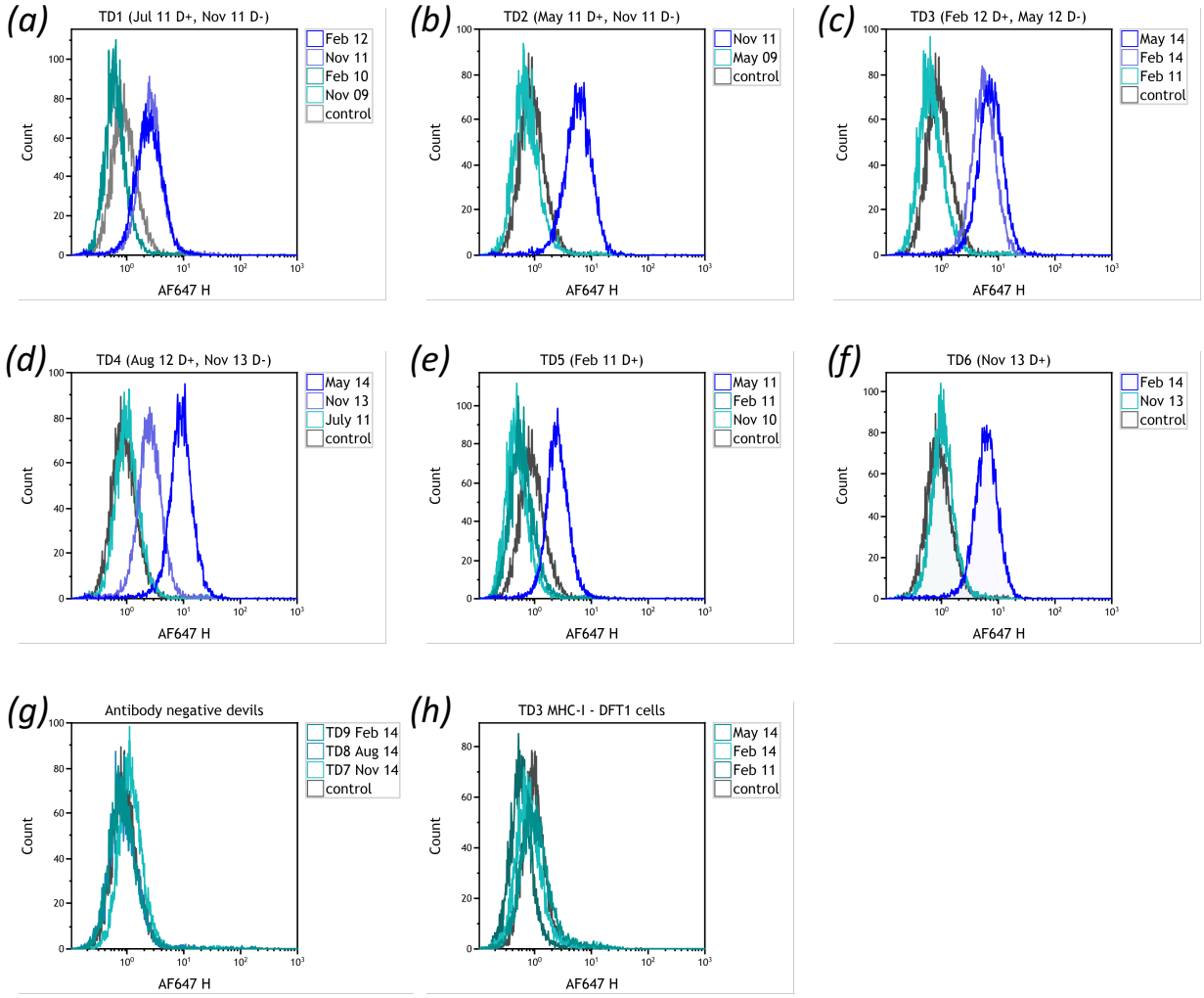


Figure 4.1. Flow cytometric analysis of anti-DFTD antibody responses. (a to f) IgG serum antibody results of TD1 to TD6 against MHC-I^{+ve} DFTD cells compared to negative control. In brackets are the dates each devil was first observed with DFTD (D+) and when the tumour was no longer present (D-); (g) representative results from 3 of the 46 devils that had no serum antibody; (h) negative results of TD3 for serum IgG against MHC-I^{-ve} DFTD cells, representative of TD1 to 6. Control = serum from wild devil from DFTD-free island.

Table 4.5. Immunohistochemistry for DFTD biopsies. Columns show number of positive staining cells per high powered field (HPF) counted.

Animal ID	CD3+ tumour infiltrating T cells/ HPF ± SD	MHCII+ tumour infiltrating cells/ HPF ± SD
TD 416	0	/
TD 417	1± 1	/
TD 421	0	/
TD 433	0	/
TD 439	0	/
TD 455	0	/
TD 473	0	/
TD 3	4±2	4± 2
TD 5	20± 2	18±2

NB All biopsies came from devils which also had serum samples assessed for anti-DFTD antibody. Only TD3 and TD5 had serum antibody against DFT1 cells.
10 high powered fields (HPF) counted for all tumours except for TD3 (7 x HPF) and TD5 (3 x HPF) because these were the only areas for the tumours.
"/" signifies not performed

Tumour regression for TD5 and TD6 was not observed, however their tumour samples showed interesting features. At the time of TD5's seroconversion, the biopsy had tumour infiltration of MHC-II and CD3 positive cells (Fig. 4.2e,f,h,i; Table 4.5). TD6 had tumour FNA's and serum collected when first trapped and again three months later. Cells from the initial FNA were periaxin positive and faintly positive for β_2m (Fig. 4.2j and k), however three months later, coinciding with seroconversion, the periaxin positive cells were strongly positive for β_2m indicating MHC-I expression by the DFTD cells (Fig. 4.2l). Neither TD5 nor TD6 were retrapped following seroconversion. In contrast to TD3, TD5 and TD6, the tumour samples from devils without serum antibody did not show significant tumour infiltration of immune cells or β_2m staining.

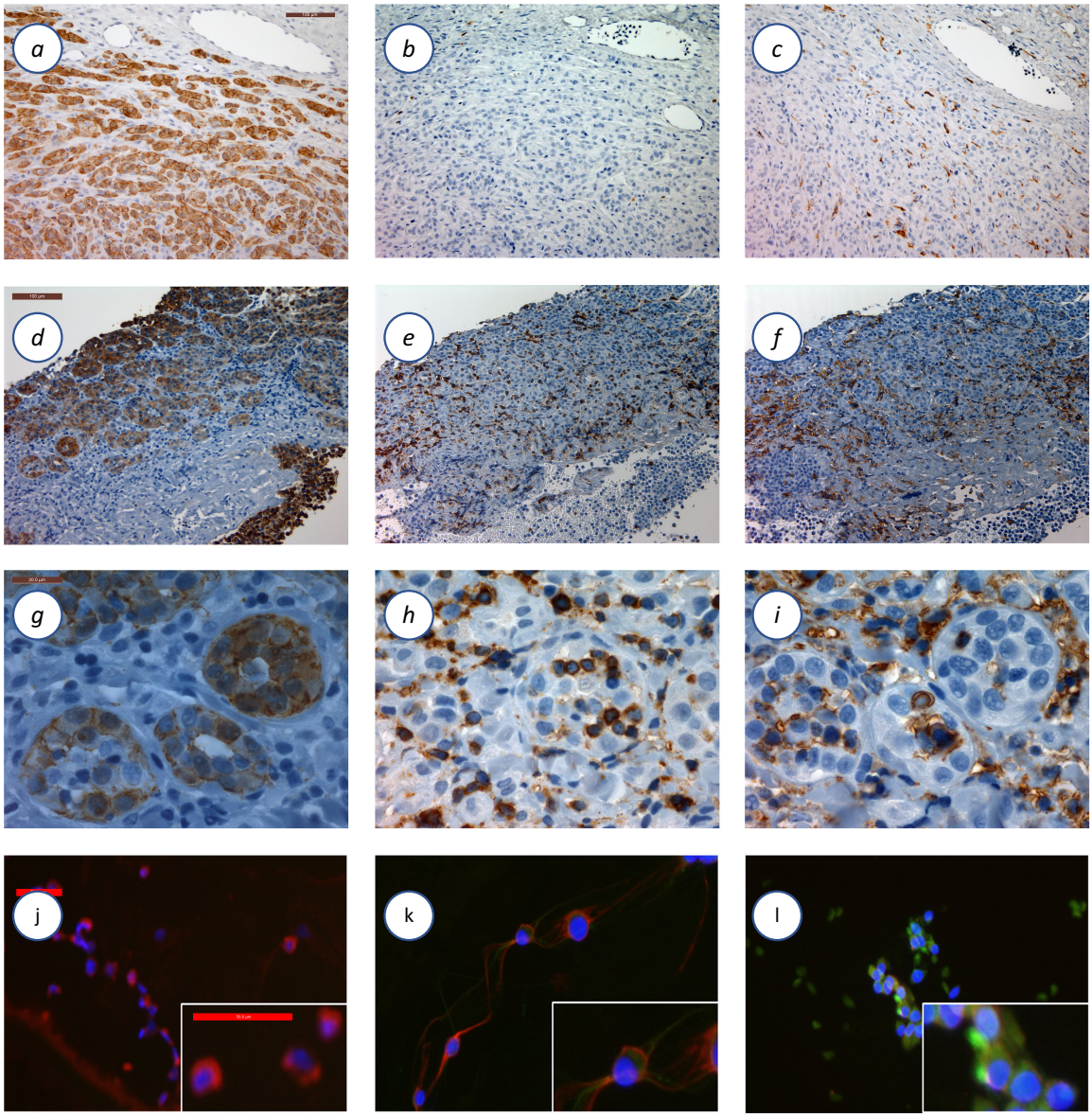


Figure 4.2. Evidence for immune cell infiltration of DFTD or MHC-I expression of DFTD cells. (a to i) IHC staining of DFTD tumour biopsies. Top and middle rows taken at 20x magnification, scale bar indicates 100 µm. Bottom row taken at 100x magnification, scale bar indicates 20 µm. Positive cells for each marker are brown; haematoxylin (blue) is the counter stain. (a,d,g) periaxin, marker for DFTD cells; (b,e,h) CD3, marker for T lymphocytes; (c,f,i) MHCII, marker for antigen presenting cells; (a to c) typical DFTD biopsy with no evidence of immune response; (d to f) tumour biopsy from TD5 showing infiltration of CD3 and MHCII positive cells throughout the tumour; (h & i) immune cells infiltrating DFTD cell clusters. (j to l) ICC of DFTD cells with periaxin (red), and β_2m (green) to identify MHC-I surface expression: (j) DFTD cells from culture; (k) DFTD FNA from TD6 collected in Nov 2013; (l) DFTD FNA from TD6 collected in Feb 2012. (ICC images taken at 20x magnification)

4.4. Discussion

The immune escape mechanisms of DFTD play a significant role in its successful transmission and tumour development. While anti-DFTD immune responses have been induced in captive devils by immunising with killed DFTD cell preparations (Kreiss et al., 2015), no convincing evidence for immune responses against DFTD had been identified in wild devils. This study reports the first evidence of anti-DFTD immune responses occurring in wild Tasmanian devils exposed to DFTD.

The serum antibodies directed against IFN-γ treated DFTD cells (MHC-I^{+ve}) found in six devils may have resulted from an initial immune response against the primary tumour and subsequent IFN-γ release. This may have up-regulated MHC-I expression on the DFTD cells, resulting in an immune response against these modified cells. The results presented here indicate that this response is initiated by infiltrating T lymphocytes, which although rare, have been documented in at least one case of DFTD and associated with tumour cell surface expression of MHC-I (Siddle et al., 2013). What caused the initial immune response in these six devils is not clear. However, the increase in MHC-I expression on DFTD cells potentially provided a mechanism for T cell mediated killing of tumour cells and ultimately tumour stabilisation or regression. Antibody production, in the form of IgG, provides confirmatory evidence that an anti-DFTD immune response had been generated. The IgG antibodies could facilitate tumour cell killing via antibody dependent cell mediated cytotoxicity.

It is not certain which epitopes on the MHC-I^{+ve} DFTD cells are recognized by the serum antibodies found in these six devils. Possible candidates are: the MHC-I molecule; the peptide presented by the MHC-I molecule; and/or other molecules that are also up-regulated by cytokine incubation but not necessarily associated with MHC-I. Identification of these epitopes will require further analysis, for example by western blotting.

Cell mediated immunity, specifically T cell, has a primary role in tumour immunity. There is however evidence for antibody production correlating with anti-tumour activity in human cases of breast and pancreatic cancer as well as in CTVT in dogs (Blixt et al., 2011, Hamanaka et al., 2003, Cohen, 1972). Although there are significant differences between CTVT and DFTD, they share characteristics of transmissibility and MHC-I down-regulation. Indeed, the development of IgG antibodies against DFTD cells may parallel what is believed to occur in

CTVT cases: after the canine tumour has established there are increased numbers of MHC-I^{+ve} CTVT cells discernable by immunohistochemistry and immunocytochemistry, and the development of serum IgG antibodies against CTVT cells occurs (Hsiao et al., 2002, Cohen, 1972). The experimentally induced CTVT's tend to regress (Hsiao et al., 2002) whereas the naturally occurring tumours seem to remain in equilibrium as locally invasive tumours with metastases being uncommon (Das and Das, 2000). It is probable that this equilibrium or regression occurs as a result of the increased MHC-I expression of the tumour cells. The consecutive tumour FNA's taken from TD6 showed increased intensity of β_2m surface staining indicative of increased MHC-I expression on the DFTD cells. Up-regulation of MHC-I, along with seroconversion occurring at that time, indicates that DFTD and CTVT may share additional characteristics of disease progression.

While there has been no observed reduction in the demographic effect of DFTD in the local population of this study, this evidence indicates that DFTD does not always escape detection by the immune system, and death may not be the inevitable outcome of infection. The naturally occurring immune responses against DFTD may enable identification of significant tumour antigens and thus advance DFTD vaccine development. If there is a heritable component to the immune response, over time selection should favour those individuals that are able to recognize the tumour, with increased survival and therefore lifetime reproductive output leading to increased representation of these devil lineages and increased survival of wild populations.

In summary, this study demonstrated a naturally occurring immune response against DFTD in this population of wild devils. The presence of anti-DFTD antibodies in the four devils with tumour regression is indicative of immune-mediated regression. The findings highlight the value of monitoring disease at the individual level where ongoing microevolutionary changes can be detected and permit evaluation of their impact on the disease trajectory and epidemic outcome at a population level.

Chapter 5

Immune mediated regression of devil facial tumour disease following immunotherapy in immunised devils

Chapter 5. Immune mediated regression of devil facial tumour disease following immunotherapy in immunised devils

5.1. Introduction

The development of a DFTD vaccine would provide an extremely useful conservation tool for efforts aimed at preventing extinction of the wild Tasmanian devil. Research toward this goal has been underway since 2006. There are many factors which make this a reasonable objective: the highly conserved morphology and genotype of DFTD (Woods et al., 2007); the ability of devils to produce both cytotoxicity and antibody responses against xenogeneic cancer cells (Brown et al., 2011); the demonstration that mice injected with DFTD cells produce an immune response against the cells and do not develop tumours (Pinfold et al., 2014); and finally, the results from preliminary DFTD immunisation trials on devils which showed that devils can produce humoral and cellular immune responses against DFTD cells (Kreiss et al., 2015).

A subsequent study demonstrated that immune mediated regression of an experimentally induced devil facial tumour is possible (paper submitted). The devil in the study was immunised with DFTD cells. She was then challenged with live DFTD cells and developed a tumour. This tumour regressed following the administration of various immunotherapies including a subcutaneous injection of live DFTD cells treated to express surface MHC-I (i.e. MHC-I^{+ve} DFTD cells). It was hypothesised that this injection of MHC-I^{+ve} DFTD cells was primarily responsible for the tumour regression.

This current study initially sought to establish whether anti-DFTD tumour responses could be induced by inactivated MHC-I^{+ve} DFTD cells. Immunisation with these cells resulted in antibody responses against DFTD. It also delayed but was not sufficient to prevent experimental tumour engraftment in all the devils. This provided the opportunity to repeat the immunotherapy used in the previous study i.e. administration of live MHC-I^{+ve} DFTD cells. Once again, this resulted in immune mediated tumour regression. This study had two outcomes. It demonstrated that inactivated MHC-I^{+ve} DFTD cells as the antigenic basis of an immunisation protocol will elicit antibody responses against DFTD. It also confirmed that DFTD tumours can undergo immune mediated regression *in vivo*.

5.2. Materials and methods

5.2.1. Tasmanian devils and biological sample collection

The Tasmanian devils were housed according to conditions described in Chapter 2.3.2. The first immunisation trial (Trial 1) used two devils. Trial 2 used a total of four devils: two for immunisation, one as an adjuvant-only control, and one as the non-immunised control for the live DFTD cell challenge. The age, sex and origin of the devils in each of the trials is summarized in Table 5.1.

Table 5.1. Age, sex and origin of the devils in each of the trials.

Devil	Age (years) at the start of the trial	Sex	Origin	Trial
Badger	7	Female	Wild born	1
Maydim	6	Female	Wild born	1
Tip	6	Male	Captive born	2
Stinky	7	Female	Captive born	2
Phil	5	Male	Wild born	2 Adjuvant control
Merrick	5	Male	Captive born	2 Non-immunised control for live challenge

The two devils in Trial 1 did not require anaesthesia for immunisation or blood collection. These procedures along with live cell challenge and therapy on the four devils in Trial 2 were performed under general anaesthesia according to Chapter 2.3.6.

Up to 10 ml of blood was obtained from the jugular vein, with up to 4 ml placed into clot activating tubes for serum analysis, and the remainder into lithium heparin anticoagulant tubes for separation of peripheral blood lymphocytes (PBL's) for cytotoxicity assays. See Chapter 2 for blood collection (2.3.3), and serum and PBL separation (2.2.2 and 2.2.3).

Tumour biopsies were collected using sterile 4 mm disposable biopsy punches (Kai Medical). Biopsies were divided with a scalpel blade and one portion placed into 1 ml 10% neutral buffered formalin and the other into 1 ml RNAlater.

5.2.2. Cell culture and preparation of DFTD cells for immunisation and challenge

The immunisation protocols for Trial 1 and Trial 2 are summarized in Table 5.2. See Chapter 2 for full details on cell culture (2.2.4) and immunisation preparation (2.2.9).

Table 5.2. Immunisation protocols for Trials 1 and 2.

Time	Trial 1 (two devils)	Trial 2 (two devils and one adjuvant-only control*)
Week 0	3 x 10^7 MHC-I^{+ve} DFTD cells sonicated	2 x 10^6 MHC-I^{+ve} DFTD cells irradiated
Week 2	/	2 x 10^6 MHC-I^{+ve} DFTD cells irradiated
Week 4	3 x 10^7 MHC-I^{+ve} DFTD cells sonicated	2 x 10^6 MHC-I^{+ve} DFTD cells irradiated
Week 8	2 x 10^6 MHC-I^{+ve} DFTD cells irradiated	2 x 10^7 MHC-I^{+ve} DFTD cells sonicated
Week 12	2 x 10^6 MHC-I^{+ve} DFTD cells irradiated	/
1st Booster 4 months after last immunisation	2 x 10^6 MHC-I^{+ve} DFTD cells irradiated (both devils)	/
1st Booster 6 months after last immunisation	/	2 x 10^6 MHC-I^{+ve} DFTD cells irradiated both devils
2nd Booster 7 months after previous booster	2 x 10^6 MHC-I^{+ve} DFTD cells irradiated (one devil only)	/

Final cell suspensions were in 1ml PBS and the following adjuvants added to each immunisation: ISCOMATRIX 50 µl; polyI:C 100 µg; CpG 1585 50 µg and CpG 2395 50 µg.

*The adjuvants were added to 1 ml PBS for the adjuvant-only control devil.

"/" indicates not performed

Live tumour challenge:

One immunised devil (Maydim) was challenged with a single dose of 25,000 live DFTD cells (cell line C5065) resuspended in 0.25 ml of PBS injected subcutaneously into the rump. Two immunised devils (Tip and Stinky) and the non-immunised control (Merrick) were challenged with 100,000 live DFTD cells in 1 ml of PBS into the left-hand side of the rump and 25,000 live DFTD cells into the right-hand side of the rump. The areas of injection were shaved for easier visualization and to mark the location. The devils were examined monthly under anaesthesia for evidence and measurement of tumours.

5.2.3. Immune response analysis

See Chapter 2 for the flow cytometry procedure to detect serum antibodies (2.2.7), and the cytotoxicity assay method (2.2.8) to identify cell mediated immune responses. For flow cytometry analysis, the median fluorescence intensity ratio (MFIR) was used to classify the antibody responses. The MFIR is the median fluorescence intensity (MFI) of DFTD cells labelled with immune serum divided by the MFI of DFTD cells labelled with pre-immune serum. The responses were considered:

Negative: MFIR < 1.5 times the pre-immune response
Low: MFIR 1.5 to 2 times the pre-immune response
Medium: MFIR 2 to 4 times the pre-immune response
High: MFIR > 4 times the pre-immune response

Tumour biopsies were processed and stained via immunohistochemistry with anti-human CD3, anti-human periaxin, anti-devil CD4 and anti-devil CD8. See Chapter 2.2.1 for histology and immunohistochemistry methods.

5.3. Results

Four devils were immunised in two separate trials (Trials 1 and 2) using MHC-I^{+ve} DFTD cells inactivated either by sonication or gamma radiation, plus adjuvants. All devils received four immunisations in their primary course followed by boosters. The protocols of the two trials differed in the interval between immunisations and the order that sonicated or irradiated cells were given. The aim was to determine if immunisation with non-viable MHC-I^{+ve} DFTD cells would produce a measurable immune response that would protect the devils against a live DFTD cell challenge. In Trial 2, one devil (Phil) was used as an adjuvant-only control and one devil (Merrick) as a non-immunised control for the live challenge. There were no adverse reactions identified to the immunisations.

For the devils in Trial 2, an attempt was made to assess cell mediated immune responses to the immunisations with a flow cytometry based cytotoxicity assay. The reliability of this assay was uncertain and so emphasis was placed on the DFTD-specific IgG antibody levels in devil serum to evaluate the immune responses after the immunisations in each protocol. Antibody responses were evaluated against interferon gamma (IFN-γ) treated (MHC-I^{+ve}) and untreated (MHC-I^{-ve}) DFTD cells.

Three of the immunised devils and the non-immunised control devil were later challenged with live DFTD cells. Tumours developed in two of the immunised devils and the control devil and immunotherapy was administered. Tumour biopsies were taken before and after immunotherapy, and evaluated for immune cell infiltration by immunohistochemistry. The tumour rejection and anti-DFTD antibody responses that occurred in the two immunised devils were recorded.

5.3.1. Trial 1

Protocol

Two devils (Maydim and Badger) were given a primary course of four immunisations at monthly intervals. The first two immunisations were sonicated DFTD cells that had been treated with IFN-γ to up-regulate MHC-I. The third and fourth immunisations were irradiated, IFN-γ treated DFTD cells. Boosters with irradiated MHC-I^{+ve} DFTD cells were given after four

months (both devils) and again after another seven months (one devil, Maydim). All immunisations/boosters included the adjuvants ISCOMATRIX™, Poly I:C and CpG.

Responses to immunisation

Both Maydim and Badger developed moderate antibody responses against IFN-γ treated MHC-I^{+ve} and untreated DFTD cells (Figs 5.1a,b). Maydim was given two booster injections of irradiated MHC-I^{+ve} DFTD cells at four and eleven months after the last immunisation but these did not appear to increase the antibody levels.

Response to live DFTD cell challenge

Badger was euthanized for age-related health reasons soon after her booster and wasn't challenged with live DFTD cells. After the second booster, Maydim was challenged with a single injection of 25,000 live DFTD cells on the rump. No tumour had developed after six months at which point she was euthanased for age-related health reasons. Medium to high antibody responses against both IFN-γ treated and untreated DFTD cells were detected after the live tumour cell challenge and similar levels identified in the post-mortem sample (Fig. 5.1c).

Trial 1 conclusion

This trial showed that inactivated MHC-I^{+ve} DFTD cells when used in combination with adjuvants could elicit antibody responses against DFTD cells. It suggested that an immunisation protocol could delay and possibly prevent experimental DFTD tumour engraftment.

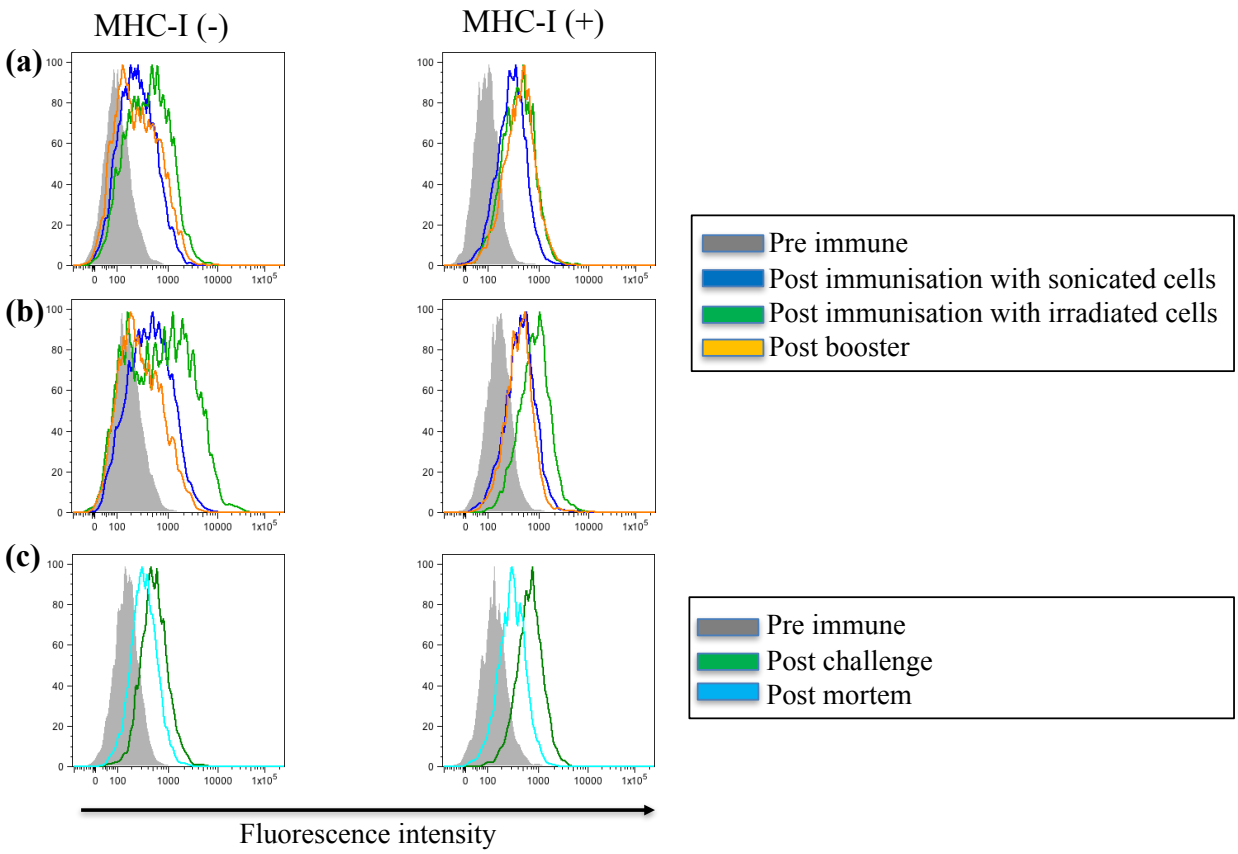


Figure 5.1. Trial 1 antibody response in Maydim and Badger after immunisation. (**a**) Maydim developed medium to high antibody responses against both MHC-I^{+ve} and MHC-I^{-ve} DFTD cells after immunisation with sonicated and irradiated MHC-I^{+ve} DFTD cells, and a booster with irradiated MHC-I^{+ve} DFTD cells. (**b**) Badger produced medium to high levels of antibodies against both MHC-I^{+ve} and MHC-I^{-ve} DFTD cells following the same immunisation protocol as Maydim. Badger was euthanased before DFTD tumour cell challenge. (**c**) For Maydim, antibody levels were maintained after challenge with live MHC-I^{+ve} DFTD cells and persisted until post-mortem 189 days later. No tumour development was detected after challenge.

5.3.2. Trial 2

Protocol

The second trial attempted to shorten the immunisation protocol by giving three irradiated MHC-I^{+ve} DFTD cell preparations at fortnightly intervals. Two devils (Tip and Stinky) received this protocol. Only a minimal antibody response resulted in Tip, so a fourth immunisation (sonicated MHC-I^{+ve} DFTD cells) was given to both devils one month after the third. A booster with irradiated MHC-I^{+ve} DFTD cells was given six months later. All immunisations/boosters were in combination with ISCOMATRIX™, Poly I:C and CpG adjuvants.

Responses to immunisation

Tip did not produce antibodies after receiving irradiated cells, but medium antibody responses against both IFN-γ treated and untreated DFTD cells were evident after receiving the sonicated IFN-γ treated DFTD cells. The booster did not increase the antibody response (Fig. 5.2a). Stinky produced medium/high antibody responses against both IFN-γ treated and untreated DFTD cells after receiving the irradiated IFN-γ treated DFTD cells. The antibody response increased after the fourth immunisation with sonicated IFN-γ treated DFTD cells (Fig. 5.2b).

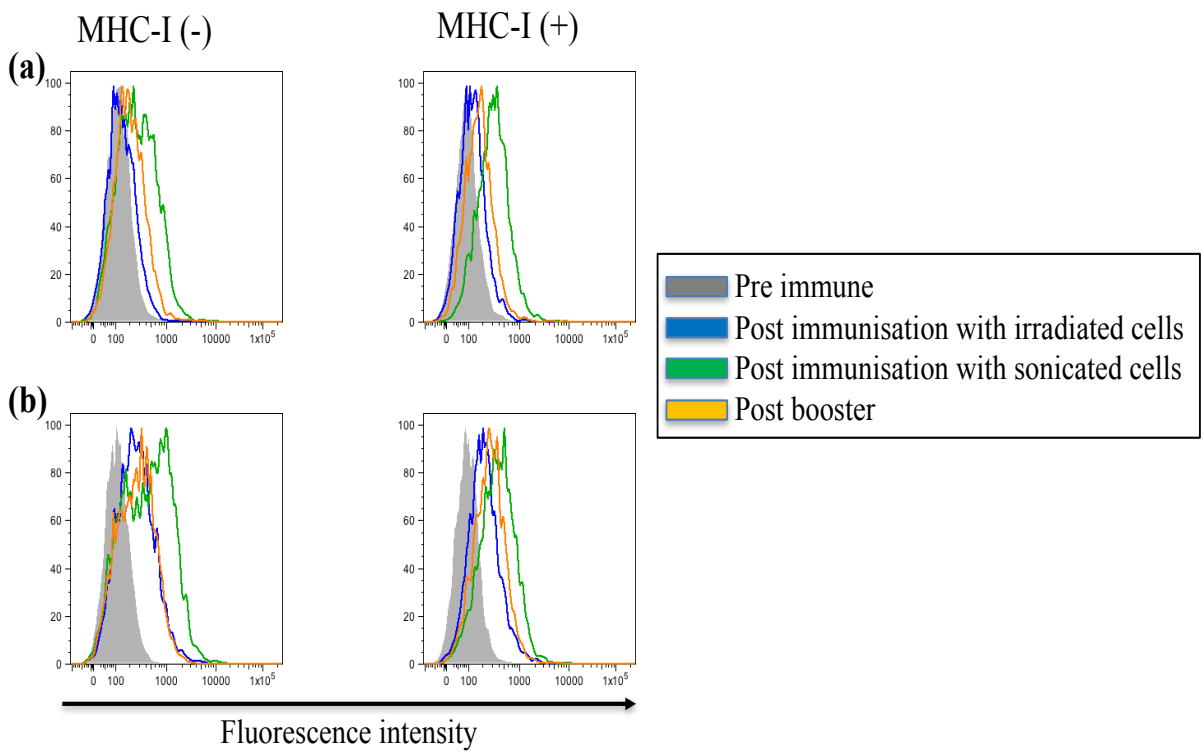


Figure 5.2. Trial 2 antibody responses in Tip and Stinky after immunisation (a) Tip: Antibody responses against DFTD cells were not detected after immunisation with irradiated MHC-I^{+ve} DFTD cells. Immunisation with sonicated MHC-I^{+ve} DFTD cells induced medium levels of antibodies against both MHC-I^{-ve} and MHC-I^{+ve} DFTD cells. A booster with irradiated MHC-I^{+ve} DFTD cells did not increase the antibody levels. (b) Stinky: Medium levels of antibody against both MHC-I^{+ve} and MHC-I^{-ve} cells were observed after immunisation with irradiated MHC-I^{+ve} cells. Antibody levels increased after immunisation with sonicated MHC-I^{+ve} DFTD cells. Booster with irradiated cells did not alter the level of antibody.

Cytotoxicity assays were performed for both devils after each immunisation, and for the adjuvant-only control devil (Fig. 5.3). The cytotoxicity assay results were deemed unreliable if spontaneous cell death in the control wells was > 30%. There was evidence for cytotoxicity for all three devils prior to immunisation. Cytotoxicity appears to be the highest two weeks following the first immunisation, but this also occurred with the control devil. After the third immunisation, all three devils showed cytotoxicity results similar to their pre-immune samples.

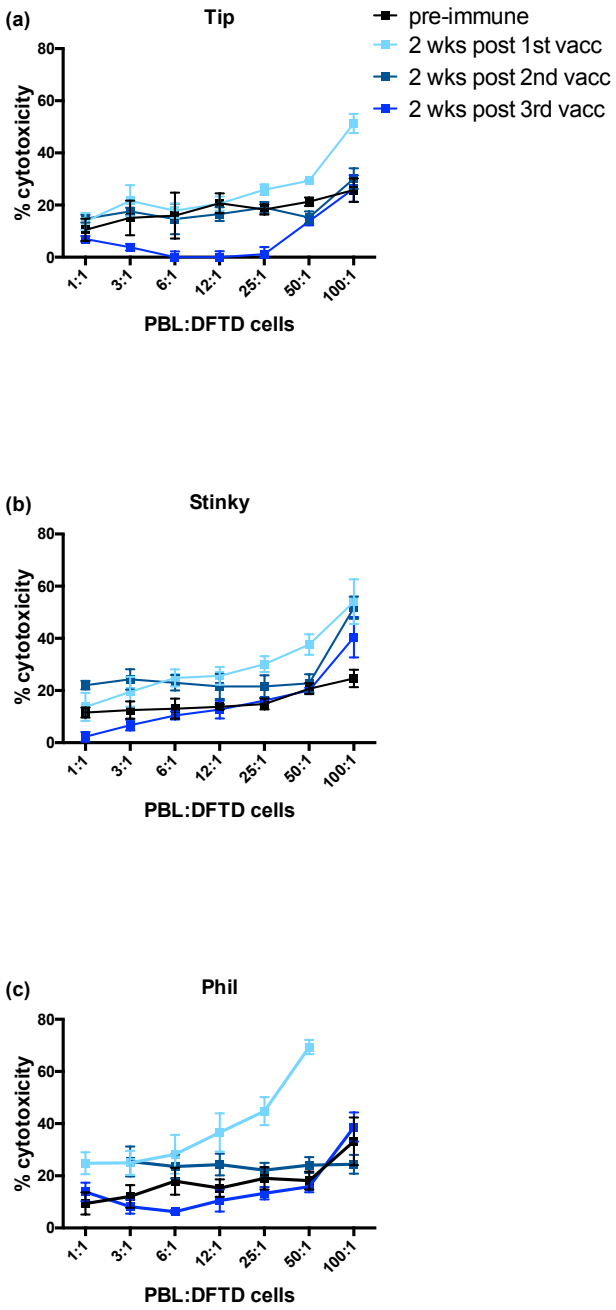


Figure 5.3. Cytotoxicity curves for (a) Tip, (b) Stinky and (c) Phil (adjuvant-only control) showing killing of DFTD cells (y axis) by different ratios of PBLs (x axis).

Live DFTD cell challenge, immunotherapy and responses

Both Stinky and Tip were challenged 28 days after the booster with 25,000 live DFTD cells on the right hand side (RHS) of the rump and 100,000 live DFTD cells on the left hand side (LHS) of the rump. The higher dose was used to exclude the possibility that tumour development required more than 25,000 cells.

DFTD tumours developed at both inoculation sites 90 days after the challenge in Tip, and at both inoculation sites 120 days after challenge in Stinky. Immunohistochemistry of the LHS tumours of both devils revealed limited immune cell infiltration (Figs 5.4a, 5.5a). When these tumours reached between 20 cm^3 and 30 cm^3 in volume the devils were subcutaneously injected in the interscapular region with 10^8 live IFN-γ treated MHC-I^{+ve} DFTD cells.

Following immunotherapy, the original tumours in both devils increased in size then one week later both tumours began to regress. A month after the immunotherapy, biopsies of the LHS tumour (Tip) and LHS and RHS tumours (Stinky), revealed moderate numbers of MHC-II+ cells at the periphery of the tumour and some cells within the tumour. A large number of CD3+ cells showed a similar distribution to the MHC-II+ cells, with CD8+ cells more abundant than CD4+ cells (Figs 5.4b, 5.5b).

Antibody levels against both IFN-γ treated (MHC-I^{+ve}) and untreated (MHC-I^{-ve}) DFTD cells in both devils increased following immunotherapy (Figs 5.6 a,b). In both devils neither the LHS or RHS tumours were palpable 70 days after treatment commenced. For Stinky, the live MHC-I^{+ve} DFTD cells that were used for immunotherapy developed into a small tumour. This tumour did not increase in volume after it had reached 10 cm^3. MHC-II+ and CD3+ cells could be found throughout the tumour.

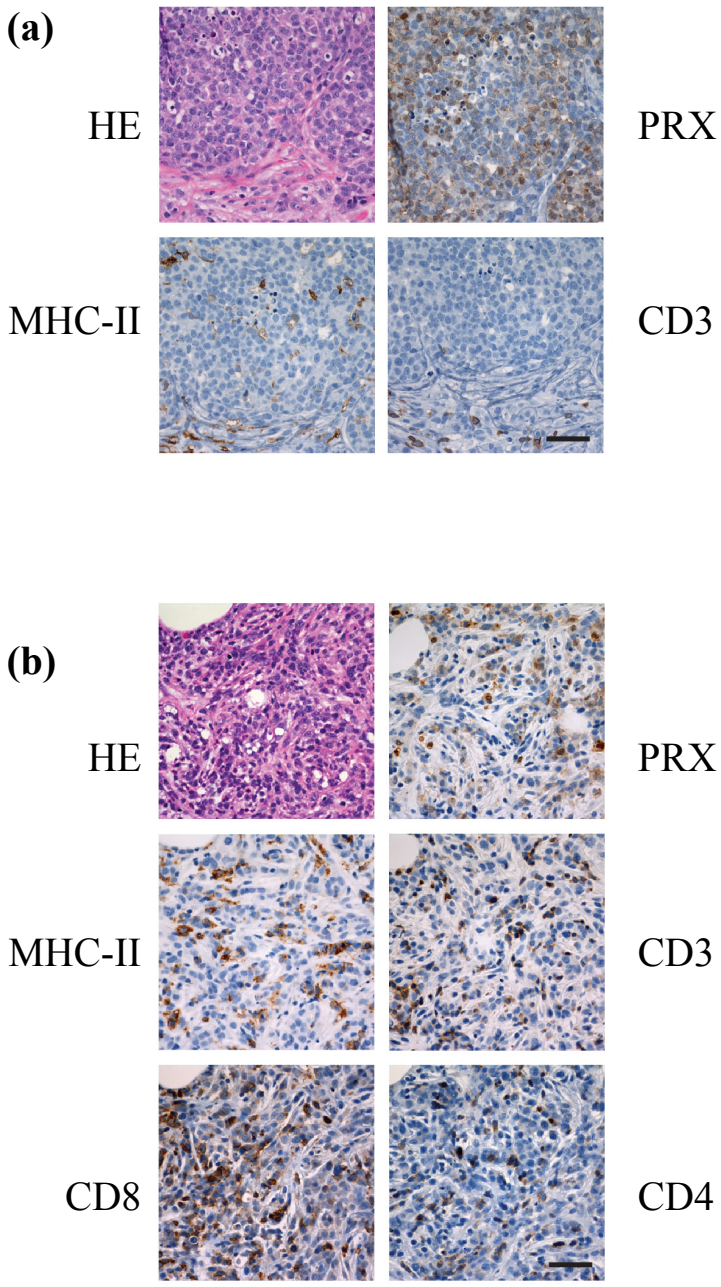


Figure 5.4. Tip. Immunohistochemistry of tumours following challenge with live DFTD cells, and after immunotherapy with live MHC-I^{+ve} DFTD cells. (a) DFTD tumours developed at both sides (left hand side – LHS, and right hand side - RHS of the rump). Representative histology of a biopsy from the LHS tumour taken 14 weeks after challenge shows very poor immune cell infiltration. Standard haematoxylin and eosin (HE) staining and immunohistochemical labelling using anti-periaxin (PRX) antibody and anti-MHC-II and anti-CD3 antibodies. Scale bar, 50 µm. (b) Following immunotherapy, tumour regression correlated with strong immune infiltration of MHC-II+ cells and CD3+ cells with CD8+ cells more abundant than CD4+ cells. Biopsy taken 4 weeks after therapy. Scale bar, 50 µm.

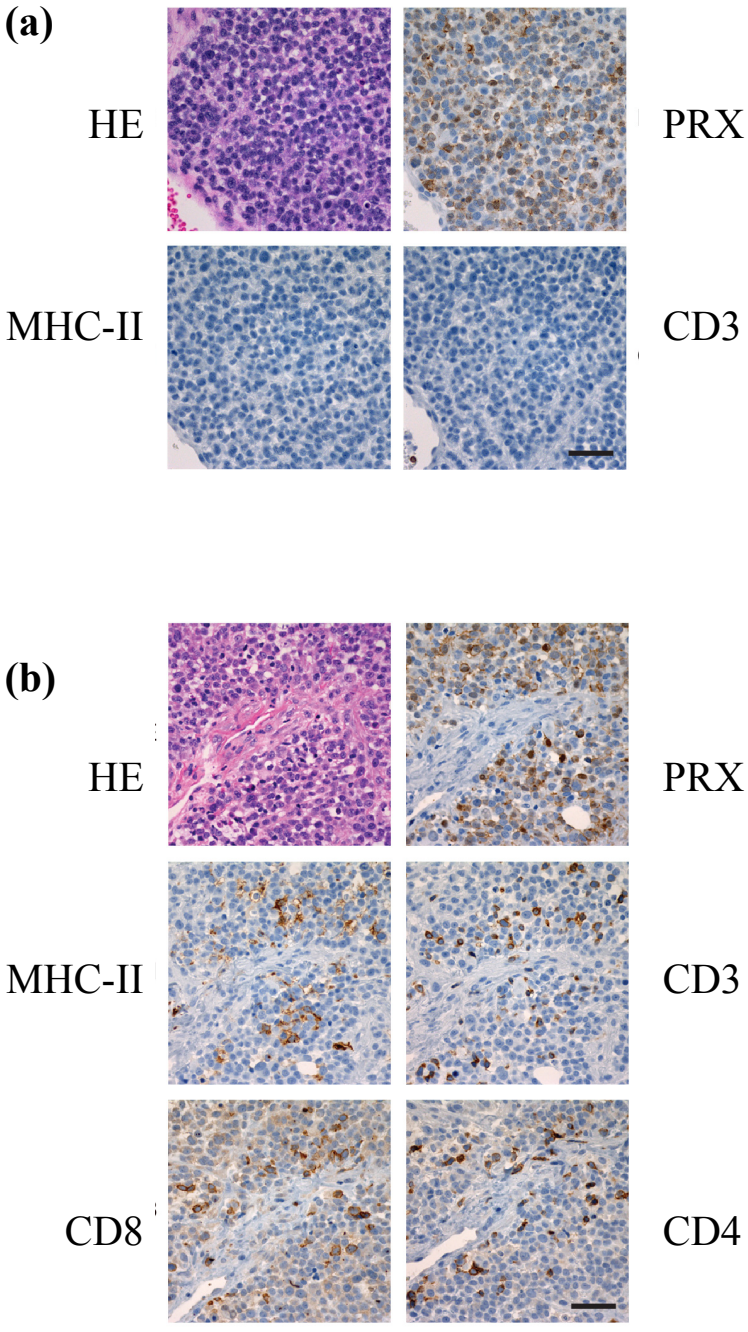


Figure 5.5. Stinky. Immunohistochemistry of tumours following challenge with live DFTD cells, and after immunotherapy with live MHC-I^{+ve} DFTD cells. (a) DFTD tumours developed at both sides (left hand side – LHS, and right hand side - RHS of the rump) of challenge with no evidence of immune cell infiltration in a biopsy of the LHS tumour taken 20 weeks after challenge. Standard haematoxylin and eosin (HE) staining and immunohistochemical labelling using anti-periaxin (PRX) antibody and anti-MHC-II and anti-CD3 antibodies. Scale bar, 50 µm. (b) Tumour regression correlated with strong immune infiltration of MHC-II+ cells and a similar distribution of CD3+ cells. CD8+ cells were more abundant than CD4+ cells. Biopsy taken 4 weeks after the immunotherapy. Scale bar, 50 µm.

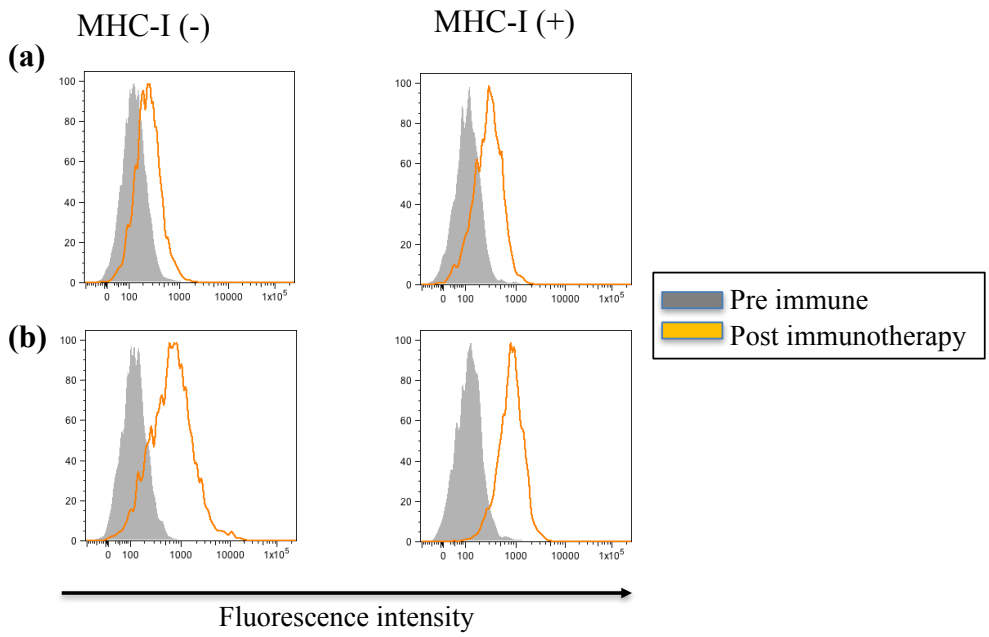


Figure 5.6. Antibody responses following immunotherapy. (a) Tip. Low to medium antibody responses against IFN-γ treated (MHC-I^{+ve}) and untreated DFTD cells were evident after immunotherapy, (b) Stinky. Medium to high antibody responses against IFN-γ treated (MHC-I^{+ve}) DFTD cells and untreated DFTD cells were evident after immunotherapy.

Adjuvant-only control devil

During Trial 2, Phil received five injections of the adjuvant components only (i.e. excluding DFTD cells) and did not produce detectable anti-DFTD antibodies (Fig. 5.7a). As with Tip and Stinky, cytotoxicity assays were performed after each adjuvant injection i.e. cytotoxicity assays were performed on all three devils at the same time so that post immunisation assay results for Tip and Stinky could be compared to their pre-immune results but also to Phil, the adjuvant-only control (Fig. 5.3). This devil was not challenged with live DFTD cells.

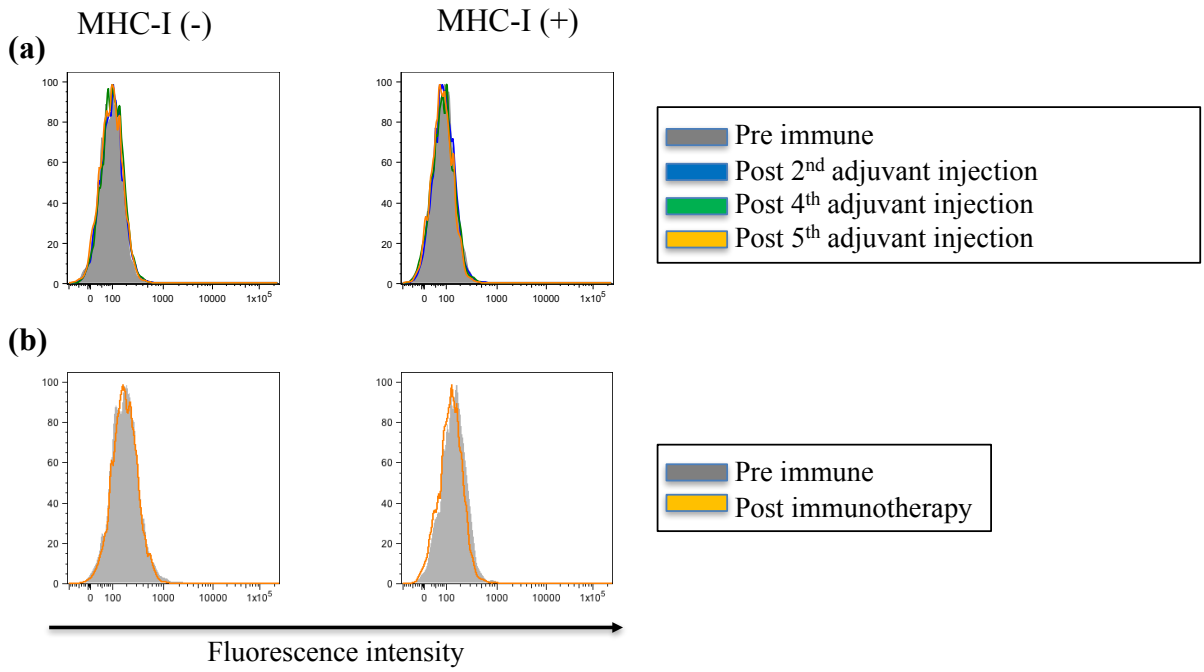


Figure 5.7. Antibody responses of (a) adjuvant-only devil (Phil) and (b) non-immunised control (Merrick) showing no antibody against DFTD cells after the intervention in either devil.

Non-immunised control devil

Merrick was not immunised and did not receive adjuvant. He was challenged with live DFTD cells in the same manner as Stinky and Tip and after 40 days, tumours were palpable on both the LHS and RHS of the rump. The tumours continued to grow, with no indication of immune cell infiltration (Fig. 5.8a).

When the tumour reached approximately 10 cm^3 in volume, the devil was subcutaneously injected in the interscapular region with 10^8 live IFN-γ treated MHC-I^{+ve} DFTD cells. A tumour developed at the immunotherapy site. Biopsies taken 28 days after the immunotherapy showed well established and encapsulated tumours. A few foci of MHC-II+ cells appeared in the periphery of the tumours with very few within the tumour. Very few CD3+ (either CD4 and/or CD8) T cells were found within or surrounding the tumour (Fig. 5.8b) and there was no evidence for antibody production (Fig. 5.7b). Due to the progression of tumour size, this devil was euthanased.

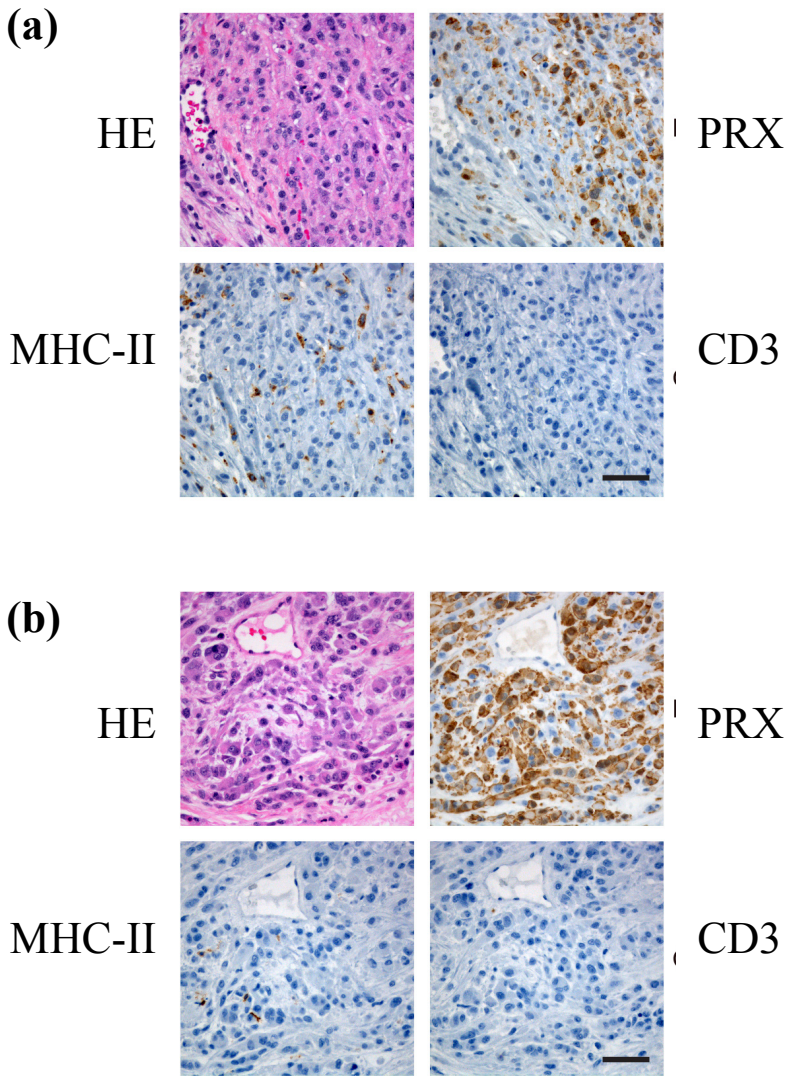


Figure 5.8. Merrick. Immunohistochemistry of tumours following challenge with live DFTD cells, and after immunotherapy with live MHC-I^{+ve} DFTD cells. (**a**) DFTD tumours developed at both sides (left hand side – LHS, and right hand side - RHS of the rump) of the challenge. A biopsy of the LHS tumour taken 10 weeks after challenge shows scattered MHC-II+ cells within the tumour and very occasional CD3+ cells. Standard haematoxylin and eosin (HE) staining and immunohistochemical labelling using anti-periaxin (PRX) antibody and anti-MHC-II and anti-CD3 antibodies. Scale bar, 50 µm. (b) Biopsy of the LHS tumour taken 4 weeks after therapy. PRX shows well established DFTD tumours with virtually no immune cell infiltration. Scale bar, 50 µm.

Trial 2 conclusion

The protocol used in this second trial was able to delay DFTD engraftment and the subsequent immunotherapy induced tumour regression.

5.4. Discussion

The effect of DFTD on the wild devil population has been devastating with declines of up to 90% in some locations (McCallum et al., 2007). A vaccine to protect against DFTD could help ensure the survival of the species in the wild. An effective vaccine must induce an immune response against the cancer cells and show evidence of efficacy *in vivo*. This study showed that DFTD cancer cells can be targeted *in vivo* by the Tasmanian devil's immune system. This was done by using sonicated and irradiated MHC-I^{+ve} DFTD cells as the antigenic basis for two immunisation trials. The protocols in each trial differed slightly but both resulted in specific antibodies against MHC-I^{+ve} and MHC-I^{-ve} DFTD cells in all four devils. This suggests that immunisation with sonicated and irradiated MHC-I^{+ve} DFTD cells in the presence of multiple adjuvants promotes anti-DFTD responses.

The immunisation strategy may have protected one devil (Maydim, Trial 1) from DFTD development following challenge with the tumour cells. The DFTD tumours that developed in two immunised devils (Tip and Stinky, Trial 2) were delayed in onset compared to the tumour in the non-immunised control. Both these devils had antibodies to untreated (MHC-I^{-ve}) and treated (MHC-I^{+ve}) DFTD cells. It is logical that a protective a vaccine should induce an antibody response against MHC-I^{-ve} DFTD cells since these cells reflect the lack of surface MHC-I on wild-type tumour cells. However, a role for antibody recognizing MHC-I^{+ve} DFTD cells was implied in wild devils that had histories of tumour regression (Chapter 4). It has been hypothesised that once an immune response is initiated, production of inflammatory cytokines e.g. IFN-γ within the tumour is likely to up-regulate MHC-I expression, allowing allorecognition to occur (Chapter 4) (Siddle et al., 2013).

Cell mediated immune responses to the immunisations in the devils from Trial 2 were based on a flow cytometry cytotoxicity assay. This was used because the chromium based assay used in previous trials (Kreiss et al., 2015) was no longer available. In this trial, cytotoxicity curves were evident in pre-immune samples which was not consistent with the chromium based assay

results (Kreiss et al., 2015, Brown et al., 2011). Similarly, the adjuvant-only devil produced cytotoxic responses. This meant any cytotoxicity demonstrated by the immunised devils could not be confidently attributed to the immunisations. Other factors that may have contributed to cell death include overcrowding and other experimental conditions such as target cell fragility. Although cell mediated immune responses to the immunisations could not be reliably demonstrated, these were subsequently shown in tumours that developed after live challenge.

The development of DFTD tumours in two of the immunised devils that were subsequently challenged with live DFTD cells provided an opportunity to treat these devils with immunotherapy and evaluate an anti-tumour immune response *in vivo*. The immunotherapy given to these devils was a therapy administered in 2012 to a devil with a similar history i.e. that devil had been immunised with DFTD cell preparations and challenged with live tumour cells which resulted in tumour development. The devils were injected with live DFTD cells that had been cultured *in vitro* with IFN-γ to up-regulate MHC-I. This particular therapy took advantage of the potential strong allogeneic responses induced by MHC incompatibilities. Following tumour growth and subsequent to immunotherapy, infiltration of CD4+ and CD8+ cells occurred in the tumours in both Stinky and Tip, and in the 2012 devil's tumour. This co-incided with tumour regression. In contrast, the tumour in the non-immunised control devil continued to grow with no evidence of a cell mediated or antibody response. It is worth noting here that the only antibody response of the 2012 devil occurred subsequent to the immunotherapy. This devil developed high levels of antibody against MHC-I^{+ve} but none against MHC-I^{-ve} DFTD cells. In short, these three cases provided the first monitored observations of DFTD tumour regression.

The different protocols in Trials 1 and 2 of this study may have influenced the antibody responses and the outcomes of the live challenge, although the small sample size makes this speculative. The protocols varied in the order that sonicated or irradiated cells were given, and in the timing between immunisations. The rationale for using irradiated cells was to present whole cells, albeit modified, to the immune system. Irradiated cells given at fortnightly intervals did not promote a robust immune response in the two devils in Trial 2. Both devils developed a higher antibody response following immunisation with sonicated cells one month later. Whether it is useful to "prime" the immune system with a larger number (2×10^7 in this study) of sonicated cells that present both intracellular and cell surface antigens to the immune system, before delivering the whole cells, is suggested by this study but not clear given the

small sample size. A shorter immunisation protocol would of course be more practical, but results from this study suggest the longer protocol from Trial 1 is more effective.

There were two key conclusions drawn from this study. Firstly, that inactivated MHC-I^{+ve} DFTD cells used as the antigenic basis for DFTD immunisations resulted in antibody responses against DFTD in all four devils. The DFTD cells used as the antigenic basis for the immunisations in both trials in this study were treated with inflammatory cytokines to up-regulate expression of surface MHC-I. The cells in some of the immunisations were subsequently treated with gamma radiation. The primary purpose of the radiation was to make the cells non-viable, but radiation can also increase the immunogenicity of the cells. This has been demonstrated with radiation resulting in the increased expression of surface MHC class I/II antigens and ICAM-I molecules (Chiriva-Internati et al., 2006), and cell surface translocation of calreticulin which promotes phagocytosis (Obeid, Panaretakis et al. 2007).

The combination of cytokine incubation and gamma radiation has been shown to have an additive effect with respect to the up-regulation and expression of cell surface antigen (Santin et al., 1996). Aside from MHC-I, it is currently unknown which antigenic molecules on the DFTD cell surface might have increased expression following manipulation. Further studies should identify up-regulated antigenic molecules, but presumably the end result of the cytokine stimulation and radiation is a more immunogeneic cell. The caveat is that inhibitory molecules can also be up-regulated with manipulation. This has been demonstrated with the increased expression of PD-L1 on tumour cells (Flies et al., 2016) in response to IFN-γ exposure.

Along with cell manipulation, the combination of adjuvants used in these immunisations most probably contributed to the immune response. ISCOMATRIX^{TM} is a proprietary saponin based adjuvant known to elicit a broad antibody response. In combination with TLR agonists it can induce regression of established solid tumours (Silva et al., 2015). The TLR agonists used in these DFTD immunisations were CPG-1585 and CPG-2395 (both TLR9 agonists), and Poly I:C (a TLR3 agonist). It is likely this adjuvant combination engaged multiple signalling pathways that support adaptive cellular immune responses in the Tasmanian devil.

The other conclusion drawn from this study was that tumour regression was associated with prior immunisation with DFTD cells, and was immune mediated. The regression was observed

after the administration of live IFN-γ treated DFTD cells and this correlated with the cellular immune response observed at the tumour engraftment site. Proliferating DFTD tumour cells expressing MHC-I provide tumour antigens for presentation and the prior immunisation may allow presentation to memory cells, enabling more DFTD-specific T cells to be activated. There was a rise in antibody levels to DFTD cells following the immunotherapy. Remission correlated with T cell and MHC-II+ cell (potentially dendritic cell) infiltration into the DFTD tumour indicative of immune mediated regression. Likewise, increased levels of IgG anti-DFTD antibodies followed immunotherapy in the immunised devils. As IgG antibodies are T cell dependent, this provides further evidence for a T cell anti-tumour immune response. This was distinct from the non-immunised control devil that had no signs of remission with no indication of immune cell infiltration into the tumour.

These results highlight the feasibility of developing a vaccine to counter devil facial tumour disease. The immune system of the Tasmanian devil is able to mount specific humoral and cellular responses to facilitate the rejection of established tumours. DFTD cells cultured in IFN-γ up-regulate MHC-I and by incorporating preparations of these cells with adjuvants that target TLRs, immune responses against the tumour cells are consistently produced. The immunisations alone in this study did not completely prevent tumour engraftment, indicating the need for stronger protective immune responses. This will require further research into the mechanisms involved in DFTD rejection. Likewise, identifying DFTD's immune escape strategies will highlight opportunities for blocking them. Pursuing these research avenues will improve the likelihood of effecting immune control of DFTD.

Chapter 6

DFTD immunisation trial on Tasmanian devils prior to their wild release

Chapter 6. DFTD immunisation trial on Tasmanian devils prior to their wild release

6.1. Introduction

Devil facial tumour disease (DFTD) immunisation trials on small numbers of captive devils have been carried out since 2006. These trials have provided key insights to the devils' immune responses against DFTD (Chapter 5), (Kreiss et al., 2015). However, their limitations include small sample sizes and senescent individuals. There is also uncertainty surrounding how well the experimental DFTD challenge replicates a natural challenge with respect to the number of DFTD cells used and their mode of delivery.

The opportunity to address these limitations arose with the implementation of the Save the Tasmanian Devil Program's (STDP) "wild devil recovery" project. The project's aim is to "trial various release methodologies to determine the most effective and successful way to release captive or semi-wild devils into the wild" (Samantha Fox pers. comms October 2016). It involves the release of devils from the STDP's captive insurance and DFTD-free Maria island populations to augment local wild devil populations that have been decimated by DFTD. The first wild release took place in September 2015 in Narawntapu National Park (NNP) in Tasmania's north. Since the devils chosen for release were held in free range enclosures for several months prior to the release date, there was the opportunity to use them in a DFTD immunisation trial. None of the devils had any exposure to DFTD prior to the trial.

The selection of the immunisation protocol for this trial was based on results of the two pilot trials (Chapter 5) that used MHC-I^{+ve} DFTD cells as the antigenic basis for the immunisations i.e. DFTD cells that had been manipulated *in vitro* to express the major histocompatibility complex class I molecule (MHC-I) on the cell surface. The protocol from Trial 1 (Chapter 5) was chosen over the Trial 2 protocol because it had resulted in higher anti-DFTD IgG antibody responses. Also, there was no tumour development for six months after experimental challenge in one devil in Trial 1 compared to tumour development in both devils in Trial 2.

There were 20 devils released into NNP in September 2015 and 19 of those were available for immunisation prior to their release. The sample size was therefore six times greater than any previous DFTD immunisation trial. The number of devils, their range of ages and fairly even ratio of sexes allowed for a more robust assessment of anti-DFTD immune responses than had

previously been possible. Along with measuring serum anti-DFTD IgG antibodies in each of the 19 devils, an attempt was made to assess the cytotoxic responses after immunisation.

Post release monitoring trips at NNP were carried out by the STDP at varying intervals for seven months following release. Serum samples were collected from the immunised devils that were trapped during these trips to assess changes to their antibody levels over time. The possibility that the devils would be exposed to DFTD following their release into NNP meant assessment of the protectiveness of the immunisation protocol might be possible.

6.2. Materials and methods

6.2.1. Tasmanian devils

There was a total of 19 devils, 11 males and 8 females, of varying ages in the immunisation trial (Table 6.1). All the devils had been selected from the captive insurance population (i.e. none were from Maria Island).

Table 6.1. Summary of age and sex of devils in the immunisation trial.

	1 year	2 years	3 years	4 years	5 years	total
Male	4	0	0	7	0	11
Female	1	1	2	3	1	8

6.2.2. Devil enclosures, trapping and blood sample collection

The devils were kept in free range enclosure (FREs) for at least eight months prior to their release. Male devils were kept in an 11 hectare FRE near Launceston, and females in a similar sized FRE near Bicheno. The devils were trapped fortnightly during the primary three month immunisation course. For the four months prior to the booster immunisation, the devils were monitored weekly with camera traps by STDP staff.

There were 15 traps set in each FRE the afternoon before procedures were performed (body weight, physical examination, blood collection and immunisation if required). The traps were baited with possum or lamb flaps and checked the following morning. Each trapped devil was transferred into a hessian sack and the handling and procedures were carried out by two

veterinarians. General anaesthesia was given in the rare event of not being able to handle the devil in the sack. Devils were released into the FRE immediately following the procedure.

Blood sample collection and general anaesthesia are described in Chapter 2.3.3 and 2.3.6. Blood was collected at each visit for serum and where possible, in lithium heparin tubes for peripheral blood lymphocyte (PBL) isolation.

Not all devils were trapped on each trip. Due to this, and to the juvenile (one year old) devils (n=5) coming later into the trial, there were some differences in the immunisation protocol given, and the blood samples available.

6.2.3. Vaccine protocol and preparation

The full immunisation protocol was the same used in Trial 1 carried out on captive devils as described in Chapter 5.2.2 and is summarised in Table 6.2.

Table 6.2. Complete immunisation protocol.

Primary course (4 immunisations given at monthly intervals)*	
Date of each immunisation	Composition of immunisations
1st (Feb 2015)	2 x 10^7 MHC-I^{+ve} sonicated cells
2nd (March 2015)	2 x 10^7 MHC-I^{+ve} sonicated cells
3rd (April 2015)	2 x 10^6 MHC-I^{+ve} irradiated cells
4th (May 2015)	2 x 10^6 MHC-I^{+ve} irradiated cells
Booster immunisations *	
Date of each booster	Composition of booster immunisations
1st (Sep 2015) pre-release	2 x 10^6 MHC-I^{+ve} irradiated cells
2nd (Feb 2016) post-release	2 x 10^6 MHC-I^{+ve} irradiated cells

*The combination of adjuvants used in each immunisation and booster was ISCOMATRIX™, Poly I:C, CpG 1585 and CpG 2395

One day of travel was required prior to the administration of the immunisations. On the morning of the travel day, sonicated preparations were taken from -80 °C, thawed and adjuvants added. Irradiated cell preparations were thawed, washed twice (with PBS, 500 g for 5 minutes), counted and resuspended in 1 ml PBS at 2 x 10^6 cells per dose, and adjuvants added.

The immunisations were kept on ice or at 4 °C for 24 hours prior to administration. Immunisations were given as a subcutaneous injection between the devils' scapulae. The immunisation preparation is described in full in Chapter 2.2.9.

6.2.4. Serum and PBL transport and isolation

Following collection, blood samples in clot activating tubes were placed in a polystyrene box with ice bricks, and samples in lithium heparin tubes were kept at ambient temperature. On return to the laboratory the same afternoon, serum and PBL's were separated as described in Chapter 2.2.2 and 2.2.3.

6.2.5. Serum antibody detection

Indirect immunofluorescence and flow cytometry to measure serum anti-DFTD IgG antibody levels was performed on the serum samples against un-manipulated DFTD cells (i.e. cells not expressing MHC-I, referred to as MHC-I^{-ve} DFTD cells) and INF-γ treated MHC-I^{+ve} DFTD cells. See Chapter 2 for MHC-I up-regulation (2.2.5) and flow cytometry (2.2.7) procedures. The median fluorescence intensity ratio (MFIR) was used to classify the antibody responses. The MFI is the median fluorescence intensity (MFI) of DFTD cells labelled with immune serum divided by the MFI of DFTD cells labelled with pre-immune serum. This ratio accounts for any background serum IgG present prior to the immunisations, as well as inevitable variations between flow cytometry experiments.

6.2.6. Cytotoxicity assays

Cytotoxicity assays to assess the killing ability of PBL's against MHC-I^{-ve} DFTD cells were set up on the evening of blood collection. The assays were kept at 37 °C overnight, and the following morning the plates were centrifuged (350 g for 3 minutes). The supernatant was collected and stored at -80 °C. The cell pellets were resuspended with 200 µl of a 1:500 dilution of propidium iodide, and the plates run through the flow cytometer to count the number of dead DFTD cells. See Chapter 2.2.8 for the cytotoxicity assay procedure and analysis.

6.2.7. Devil release into NNP and post-release monitoring

The STDP released 20 devils in total, including the 19 from the immunisation trial into NNP on 25/09/15. Monitoring trips were carried out at 2, 6, 12 and 20 weeks post release i.e. October, November and December 2015, and February 2016. The STDP's annual monitoring NNP trip took place in April 2016. Serum was collected from the immunised devils that were trapped during the monitoring trips. A booster immunisation was given to devils that were trapped in February 2016 to coincide with the mating period when most biting injuries occur and risk of DFTD transmission is highest (Hamede et al., 2008).

6.2.8. Statistical analysis

One-way ANOVAs (Figs 6.2, 6.3, 6.6) were performed to compare overall anti-DFTD IgG antibody responses at different time periods.

A four-way repeated-measures ANOVA comparing sex, age and protocol over time was performed to compare antibody responses at three time points i.e. at the end of the primary immunisation course, on the day of the booster, and two weeks after the booster (for Fig. 6.4). Tukey's post hoc analyses were performed.

One-way ANOVAs were performed using GraphPad Prism version 6 for Mac OS X, GraphPad Software, La Jolla, CA, USA, www.graphpad.com

The four-way ANOVA and Tukey's posthoc analysis were performed using R statistical software.

6.3. Results

6.3.1. Immunisation protocols

The primary immunisation protocol each devil received was dependent on trapping success, and on when the devils came into the trial (the five juveniles were late entries). Table 6.3 summarises the immunisation protocols and the number of devils that received each protocol. All the devils received a booster immunisation four months after the primary course. Table 6.4 identifies the name, sex and age of the individual devils that received each protocol.

Table 6.3. Description of immunisation protocols and the number of devils that received each protocol.

Protocol	Protocol description*	No. of devils
A	4 immunisations at 4 week intervals: 1st and 2nd: sonicated cells 3rd and 4th: irradiated cells	6
B	4 immunisations at 4 or 6 week intervals**: 1st and 2nd: sonicated cells 3rd and 4th: irradiated cells	6
C	3 immunisations at 4 week intervals: 1st and 2nd: sonicated cells 3rd: irradiated cells	3 (including 2 juveniles)
D	2 immunisations at 2 or 4 week intervals: 1st: sonicated cells 2nd: irradiated cells	4 (including 3 juveniles)

* See Table 6.2 for complete description of immunisation composition
** Two male devils (Cory and Jackson) had their 2nd immunisations 6 weeks after the 1st. There were 4 week intervals between their 2nd and 3rd, and their 3rd and 4th immunisations.
Four female devils (Cindy, Irry, Isla and Janice) had 4 week intervals between their 1st and 2nd, and 2nd and 3rd immunisations. The 4th immunisation was given 6 weeks after the 3rd immunisation.

Table 6.4. Sex and age of individual devils and the immunisation protocol each received.

Males			Females		
Immunisation protocol	Name	Age (years) in 2015	Immunisation protocol	Name	Age (years) in 2015
A	Beau	4	A	Andrea	4
A	Bruce	4	A	Heidi	3
A	Floyd	4	A	Mystique	3
B	Cory	4	B	Cindy	5
B	Jackson	4	B	Irry	2
C	Boots	4	B	Isla	4
C	Gendry	1	B	Janice	4
C	Jorah	1	D	Lysa	1
D	Chilli	1			
D	Chouffe	1			
D	Monty	4			

Immunisation protocols A-D are described in Table 6.3.

6.3.2. Anti-DFTD IgG antibody responses prior to wild release

The anti-DFTD IgG antibody responses were assessed separately against MHC-I^{-ve} and MHC-I^{+ve} DFTD cells. There were no significant differences between results for either cell type (p value between 0.176 and 0.758, data not shown), so the results presented here are for responses against MHC-I^{-ve} DFTD cells only. These responses were classified as negative, low, medium or high depending on the MFIR, where Negative = MFIR < 1.5 times the pre-immune response; Low = MFIR 1.5 to 2 times the pre-immune response; Medium = MFIR 2 to 4 times the pre-immune response; High = MFIR > 4 times the pre-immune response. Figure 6.1 shows example flow plots for low, medium and high responders.

At the end of the primary immunisation course, nine devils had medium/high responses, and only two devils had negative responses. Antibody levels in most devils dropped over the four months prior to the booster. The booster resulted in increased antibody levels in all devils, however in the adult male devils, the antibodies didn't reach the levels found after the primary immunisation course. In contrast, the booster resulted in all the female devils having antibody levels that were equal to or higher than their levels achieved after the primary course (Table 6.5).

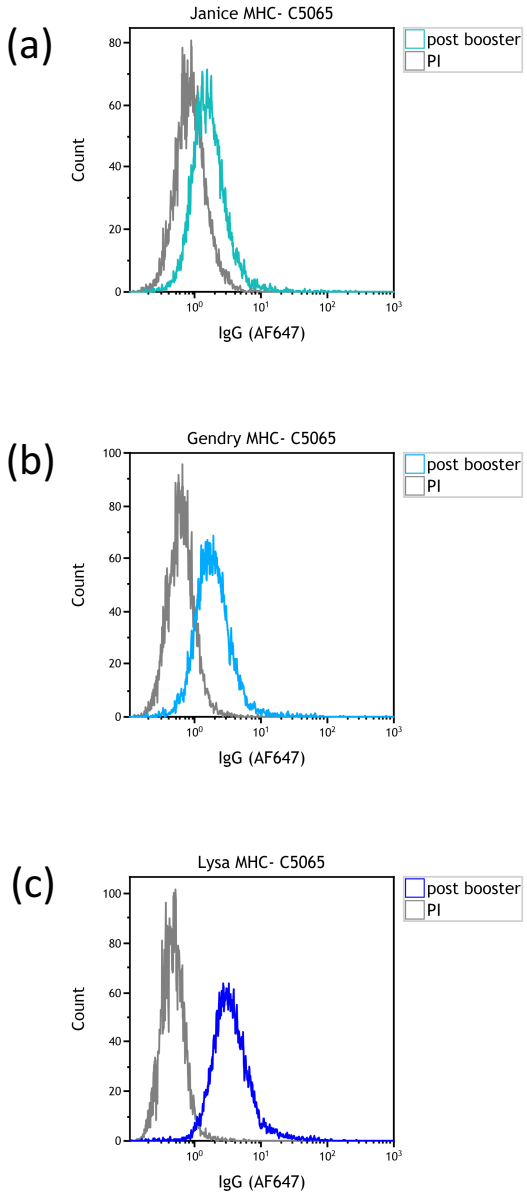


Figure 6.1. Representative flow plots of IgG antibody responses to untreated (MHC-I^{-ve}) DFTD cells two weeks post booster (coloured line) compared to pre-immune (grey line) for (a) low, (b) medium and (c) high responders.

Negative = MFIR < 1.5 times the pre-immune response; Low = MFIR 1.5 to 2 times the pre-immune response; Medium = MFIR 2 to 4 times the pre-immune response; High = MFIR > 4 times the pre-immune response. (MFIR = median fluorescence intensity ratio).

Table 6.5. Serum anti-DFTD IgG antibody levels (MFIR) for each devil at three different time points.

Response 2 weeks after primary course n=17			Response on day of booster n=19			Response 2 weeks after booster n=19		
Medium – High n = 9	**Low n = 6**	**Negative n = 2**	**Medium - High n = 4**	**Low n = 8**	**Negative n = 7**	**Medium - High n = 12**	**Low n=3**	**Negative n = 4**
Andrea 5.1	Beau 1.8	Janice* 1.3	Andrea 3	Bruce 1.7	Beau 0.7	Andrea 5	Cory 1.9	Beau 1.3
Bruce 3.6	Boots 1.6	Monty 1.0	Gendry 2.6	Cindy 1.6	Boots 1.2	Bruce 2.1	Floyd 1.7	Boots 1.4
Cory 2.3	Cindy* 1.9	Chilli**	Irry 4.6	Chilli 1.9	Cory 1.2	Chilli 3	Janice 1.9	Jackson 1.4
Gendry 3.7	Floyd 1.8	Chouffe**	Lysa 3.8	Chouffe 2	Floyd 1.3	Chouffe 3		Monty 1.1
Heidi 2.1	Jackson 1.7			Isla 1.6	Heidi 1.4	Cindy 3.2		
Irry* 2.9	Isla* 2			Janice 1.5	Jackson 1.2	Gendry 3.2		
Jorah 5				Jorah 2	Monty 1.0	Irry 5		
Lysa 6				Mystique 1.8		Heidi 2.1		
Mystique 3.4						Isla 2.1		
						Jorah 2.7		
						Lysa 6.6		
						Mystique 4.1		

Females are in red, males in black.

The number in each cell is the MFIR; Negative = MFIR < 1.5 times the pre-immune response; Low = MFIR 1.5 to 2 times the pre-immune response; Medium = MFIR 2 to 4 times the pre-immune response; High = MFIR > 4 times the pre-immune response. (MFIR = median fluorescence intensity ratio).

* These devils had Protocol B i.e. 4 immunisations, however their last serum sample for the primary course was collected on the day of their 4th immunisation which was 6 weeks after their 3rd immunisation.

** No serum sample available after primary course.

Antibody responses after each of the immunisations given in the primary course were also recorded. Figure 6.2 shows the responses for devils that received four immunisations in the primary course i.e. Protocol A or B, for which serum samples were available at each time point. Antibody levels were significantly higher after the second and third immunisations compared to after the first.

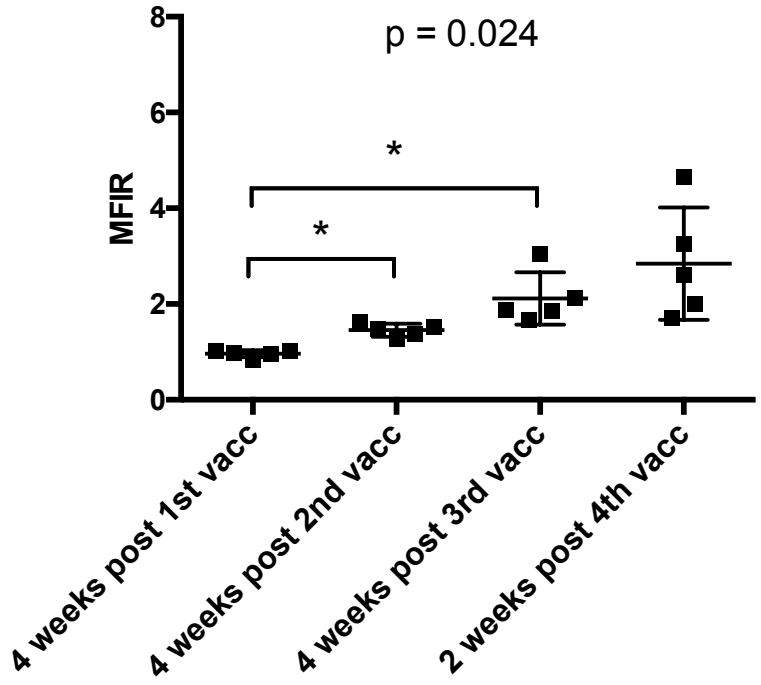


Figure 6.2. Serum anti-DFTD IgG levels (MFIR) of devils that had four immunisations in their primary course i.e. protocol A or B, Table 6.3. Only those devils for which sera samples were available at all time points are included. Statistical analysis was performed with a one-way ANOVA (with repeated measures) to produce an overall p value. Post hoc analysis identified which time points had significantly different MFIR's from each other and are indicated by *. NB pre-immune MFIR for each devil is equal to 1 and is therefore not shown.

Antibody levels of all devils at three time points (two weeks after primary course, on the day of the booster, and two weeks after the booster) were then compared. The levels on average were lowest on the day of the booster i.e. four months after the primary course. Although the average antibody level rose after the booster, this was not a statistically significant increase (Figure 6.3).

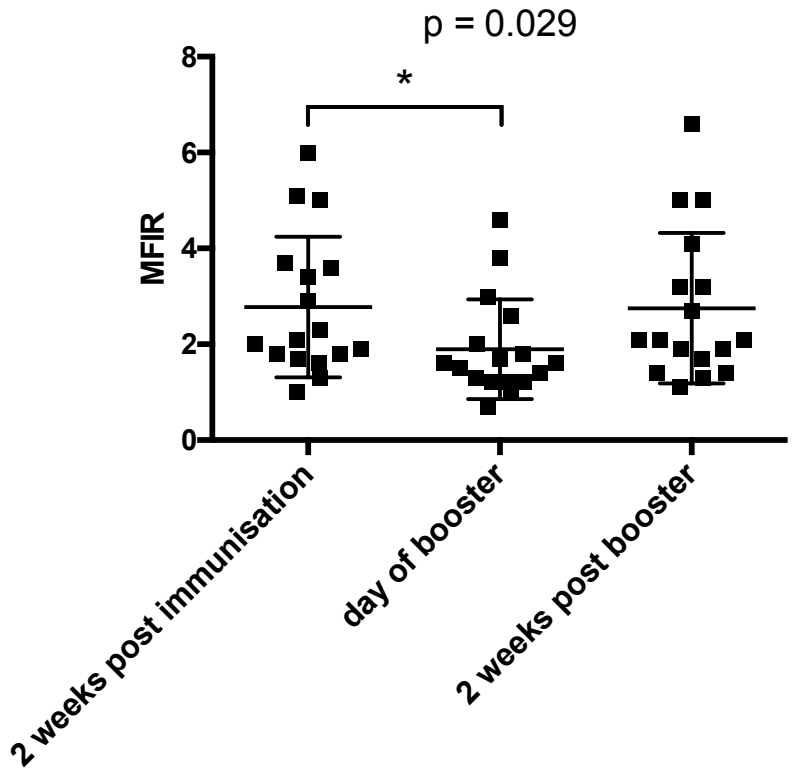


Figure 6.3. Serum anti-DFTD IgG antibody levels (MFIR) in devils for each of the three time points: end of primary immunisation course, day of booster (4 months later) and 2 weeks post booster. Statistical analysis was performed with a one-way ANOVA (with repeated measures) to produce an overall p value. Post hoc analysis identified which time points had significantly different MFIR's from each other as indicated by *. (MFIR = median fluorescence intensity ratio).

6.3.3. Effects of protocol, age and sex on antibody responses measured at three time points

The effects of protocol, age, and sex on the antibody responses measured at three time points (two weeks after primary course, on the day of the booster and two weeks after the booster) were assessed with a four-way ANOVA. The primary immunisation protocol (i.e. two, three or four immunisations) did not make a significant difference to the antibody responses (Fig. 6.4a). However, both age and sex were found to have significant effects on the antibody responses (Figs 6.4b,c). Juveniles had higher responses on average than adults, and females had higher responses than males.

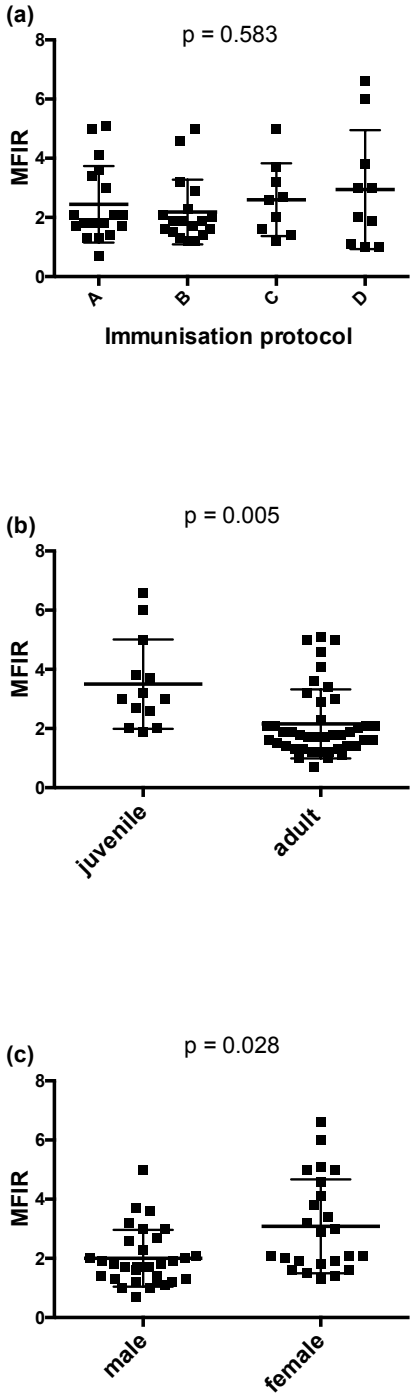


Figure 6.4. Serum anti-DFTD IgG antibody levels (MFIR) showing effect of (a) protocol, (b) age, and (c) sex. The three different time points (two weeks after primary course, on the day of the booster and two weeks after the booster) are not shown separately on each graph. Instead, the MFIR for each devil at each time point has been plotted on each graph. Protocol A = 4 immunisations at 4 week intervals, B = 4 immunisations at 4 or 6 week intervals, C = 3 immunisations at 4 week intervals, D = 2 immunisations at 2 or 4 week intervals. The p values were obtained with the four-way ANOVA analysis. See Table 6.8 for detailed ANOVA results.

6.3.4. Cytotoxicity assays

Cytotoxicity assays were performed fortnightly on the devils for which blood samples were available since not all devils were trapped each fortnight. The cytotoxicity assay results were deemed unreliable if spontaneous cell death in the control wells was > 30%. The control well cell death for the pre-immune assays of the male devils was 46.2% so only results for female devils comparing pre-immune samples to post immunisation samples are shown. Pre-immunisation cytotoxicity varied between devils, ranging from 25 to 40% at the highest effector to target cell ratio of 100:1. This level of killing was higher than in some of the post immunisation samples (Fig. 6.5).

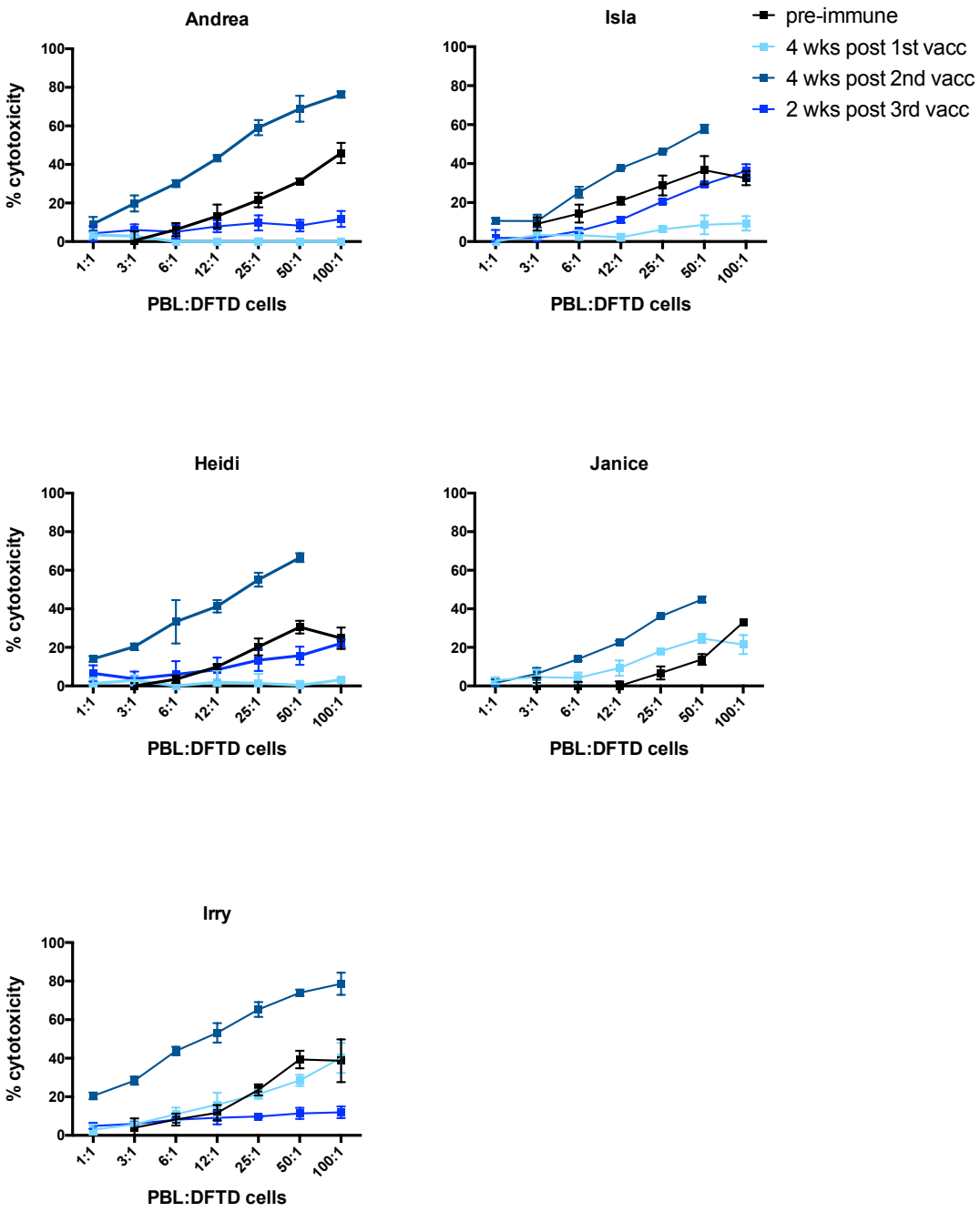


Figure 6.5. Cytotoxicity curves for five female devils showing killing of DFTD cells (y axis) by different ratios of PBLs to DFTD cells (x axis). Post immunisation cytotoxicity (coloured lines) is compared to the pre-immunisation cytotoxicity (black line).

6.3.5. Anti-DFTD IgG antibody responses after wild release

Following the release into NNP, monitoring trips were run at 2, 6, 12 and 20 weeks i.e. October, November, December 2015 and February 2016. The annual monitoring trapping trip for NNP took place in April 2016. Table 6.6 shows the dates that the trial devils were re-trapped on these trips.

Table 6.6. Post release trapping and sampling.

Post release monitoring trip	Number of immunised devils re-trapped and serum collected	Individual devils
2 weeks October 2015	6	Cindy, Heidi, Isla Beau, Boots, Bruce
6 weeks November 2015	4	Heidi Beau, Boots, Bruce
12 weeks December 2015	3	Beau, Bruce, Cory (Boots to vet but no serum)*
20 weeks booster given Feb 2015	1 (+1 at AHL)	Boots – booster at AHL, blood collected 2 weeks later Beau -booster
7 months April 2016	1	Beau

* Boots had a non-healing bite wound on his rump so received veterinary treatment and was housed at the Animal Health Laboratory (AHL) for recovery. He was re-released into NNP in March 2016.

Serum anti-DFTD IgG antibody was measured for each sample collected on the post-release trips (Fig. 6.6). Statistical analysis could only be performed on samples from the three devils that were consistently trapped. The change in antibody levels was not significant over the two month period post release. There was however a trend for antibody levels to decrease over time. Only one devil (Beau, a four year old male) was trapped on each of the post release monitoring trips. He received a booster immunisation in February 2016 after which his antibody levels increased. Only one other devil (Boots), also a four year old male, was given a second booster immunisation in February. In contrast to Beau, Boots' response after this second booster wasn't as high as his response after the first (data not shown). NB Boots was undergoing veterinary care at this time.

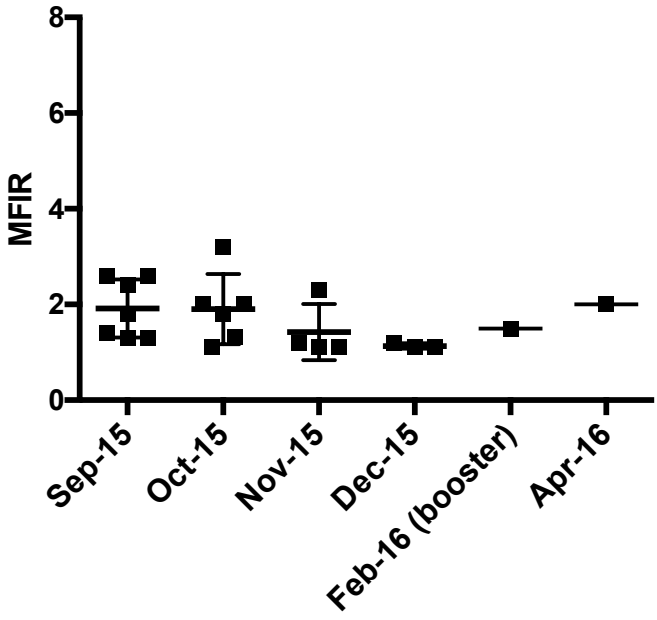


Figure 6.6. Serum anti-DFTD IgG antibody levels (MFIR) of devils collected post release compared to their post booster response in September 2015. The graph shows serum antibody levels of devils trapped on each of the post-release trips. Only 3 individual devils were trapped in both October and November and a one-way ANOVA analysis (with repeated measures) was performed, $p = 0.418$. (MFIR = median fluorescence intensity ratio).

6.3.6. Statistical analysis

One-way ANOVAs were performed to compare the antibody responses of devils during the primary immunisations course; at three time points during the immunisations before release; and of the individual devils which were trapped on both the October and November post release trips (Table 6.7).

Table 6.7. One-way ANOVA results for data analysed in figures 6.2, 6.3 and 6.6.

One-way ANOVA	F	p
Antibody responses during primary immunisation course (Figure 6.2)	8.317	0.024
Antibody responses at 3 time points before release (Figure 6.3)	4.314	0.029
Antibody responses at post release time points (Figure 6.6)	1.060	0.418

A four-way repeated-measures ANOVA comparing sex, age and protocol over time was performed to compare responses at three time points i.e. at the end of the primary course, on day of booster, and two weeks after the booster (Table 6.8).

Table 6.8. Results of four-way ANOVA comparing effects of sex, age and immunisation protocol at 3 time points

Error: ID			
	Df	F value	Pr (>F)
sex	1	6.609	0.028
age	1	12.417	0.005
protocol	3	0.682	0.583
sex:age	1	0.348	0.569
sex:protocol	1	0.234	0.639
Residuals	10		
Error: within			
	Df	F value	Pr (>F)
sex:time	1	3.811	0.061
age:time	1	0	0.984
protocol:time	3	0.82	0.494
sex:age:time	1	1.364	0.253
sex:protocol:time	1	0.163	0.69
age:protocol:time	1	1.279	0.268
Residuals	27		

6.4. Discussion

This DFTD immunisation trial was carried out on the first cohort of devils released to the wild by the STDP's "wild devil recovery" project. The collaboration brought together two "firsts" for management attempts to prevent the extinction of devils in the wild i.e. DFTD immunisation and population augmentation. The immunisation trial was carried out on a much larger number of devils than had previously been possible allowing for a comparatively robust assessment of immune responses.

There are other examples of vaccinating captive bred and/or wild populations of endangered species against fatal diseases e.g. black footed ferrets against plague and canine distemper virus (Marinari and Kreeger, 2006) (Service, 2013), kakapo against erysipelas (Gartrell et al., 2005); and Ethiopian wolves against rabies (Randall et al., 2004, Sillero-Zubiri et al., 2016). An experimental chlamydia vaccine trial has also been carried out on a wild population of koalas

in south east Queensland (Waugh et al., 2016). This DFTD immunisation trial shared aspects of these examples i.e. it focused on an endangered species facing a primary threat of disease which might be addressed by vaccination. Vaccine development is necessary to meet the ambitious long term objective of releasing devils with immunity to DFTD into the wild to augment local wild devil populations that have been decimated by the disease.

The potential exposure of released devils to DFTD meant that the benefit of this immunisation trial extended beyond vaccine development. Addressing the original threatening process in a release environment is considered requisite for successful reintroductions of individuals from captive bred populations of a threatened species (Caughley and Gunn, 1996). Despite the unproven efficacy of the immunisation protocol, an advantage of this trial was its merit as an attempt to mitigate the DFTD threat to the devils once released.

The biggest advantage of this trial with respect to DFTD vaccine development was the sample size of 19 devils. Previous immunisation studies were limited to two to four devils. The comparatively large sample size of this trial allowed for greater confidence in the assessment of anti-DFTD immune responses. It also allowed for the effects of age, sex and varying immunisation protocols to be assessed. Previous immunisation trials on captive devils have demonstrated that an immune response against DFTD is achievable. However, the representation of results with respect to the wider devil population has been uncertain due to the small sample sizes. The high number of responders in this trial suggests most devils are capable of producing an immune response against DFTD which is encouraging for vaccine development.

The four devils that had a low to negative antibody response following the booster were all adult males. This was illustrated by the analysis of results for the effects of age and sex on responses. The five juvenile devils showed on average, higher antibody responses than the adult devils. This was despite the juveniles receiving a shorter primary immunisation course (i.e. fewer doses) than the majority of adults. Female devils had higher antibody responses than males. This is in keeping with evidence that sex affects immune responses via a combination of genetic, hormonal and environmental factors (Klein and Flanagan, 2016). In general, human studies show females to have heightened immunity to pathogens, and a tendency toward higher responses to bacterial and viral vaccines than males.

Compared to conventional immunisation protocols against microbial pathogens, this DFTD immunisation protocol was long, taking seven months as it included a booster prior to the release date. Not all devils received the entire primary immunisation course, and although the number of devils receiving each different protocol was small, the results suggested that a reduced number of immunisations in the primary course did not affect the post booster antibody response. Clearly a shorter immunisation protocol would be more practical and these results suggest that similar antibody responses would be achieved with fewer immunisations.

There were three notable limitations to this trial. The first was the inability to reliably measure the cell mediated immune response to the immunsiations. With the chromium release assay used in previous trials (Brown et al., 2011, Kreiss et al., 2015) no longer available, a flow cytometry based assay was employed. The cytotoxicity curves seen in the pre-immune sample assays meant any cytotoxicity demonstrated after immunisation could not be confidently attributed to the immunisations. Likewise, the similarity of the curves for each devil on each day the assays were performed suggested the experimental conditions plus or minus the individual devil responses played a notable role in the results. Similar concerns were noted for the cytotoxicity assay results from Chapter 5. Realizing the cell death measured in the assays from this study may not provide definitive information regarding cell mediated immune response to the immunisations, the assay supernatants were stored at -80 °C. This would allow for post hoc analysis such as the presence of IFN-γ once a sensitive anti-devil IFN-γ antibody becomes available.

The other limitations were associated with the low post-release trapping success of immunised devils. The low recapture rate was partly due to the devils' likely dispersal beyond the trap lines, but also due to deaths from road traffic accidents. As a result, it was not possible to assess extended duration of antibody responses for the majority of devils. The other consequence of the low re-trapping rate was the inability to assess the protectiveness of the immunisation protocol. While a natural DFTD challenge would provide the gold standard test for immunity, the small size of the incumbent devil population in NNP (n=18) and the low DFTD prevalence (15%) (Samantha Fox pers. comms 2016) meant the probability of the released devils coming into contact with diseased devils was low. Likewise, although the incubation period of DFTD is uncertain, it seems unlikely devils would be showing clinical signs of DFTD in April 2016 (the final monitoring trip) even if they had been susceptible and exposed post-release.

Despite the limitations, this trial signifies a notable advance in DFTD vaccine research. Although it remains unknown what protection the immunisation protocol provided against a natural DFTD challenge, the serum antibodies detected in the majority of devils in response to the immunisations suggest that development of an effective DFTD vaccine is a realistic expectation. A vaccine would be a valuable conservation tool to secure the future of the wild devil population. There are suggestions that responses to DFTD are occurring in the wild. Natural immune responses against DFTD have been found in a small percentage of wild devils (Chapter 4). There is also evidence that gene selection associated with DFTD has occurred in certain populations (Epstein et al., 2016). However, there has not been a measurable reduction of the DFTD effect on these populations. DFTD has resulted in dramatic devil population decline to the point where the species is functionally extinct in certain locations (Hollings et al., 2015). Evolution is by definition a slow process, and so even assuming adequate anti-DFTD responses are evolving, the ecological impacts of a decimated devil population are profound and relying on evolution alone for population recovery at this stage seems risky. A protective DFTD vaccine would aid in a timelier restoration of a functional devil population while helping to ensure the genetic diversity of the species.

Chapter 7

Immune recognition of DFT2

Chapter 7. Immune recognition of DFT2

7.1. Introduction

Until 2015, the only clonally transmissible cancers observed in nature were Tasmanian devil facial tumor disease (DFTD) and canine transmissible venereal tumor (CTVT). That only two such cancers had ever been documented underscored the belief that transmissible cancer lineages in nature are rare and the likelihood of discovering new clones, highly improbable. However, in 2015, the leukaemia-like disseminated neoplasia in soft- shell clams was identified as a transmissible cancer (Metzger et al., 2015). In the following year, we published research confirming a second transmissible cancer affecting Tasmanian devils (Pye et al., 2016). This cancer was named devil facial tumour 2 (DFT2) in recognition of the gross similarities it shares with the first cancer, now referred to as DFT1. The original DFTD nomenclature is consequently now regarded as an umbrella term to include both tumours.

The tumours associated with DFT2 are macroscopically indistinguishable from DFT1 tumours. However, they have marked histological and cytogenetic differences. DFT2 has a Y chromosome in direct contrast to the two X chromosomes in DFT1 (Murchison et al., 2012). We found DFT2 has different alleles compared to both its hosts and DFT1 at microsatellite, structural variant and major histocompatibility complex (MHC) loci. These results indicated that DFT2 is a second independent transmissible cancer affecting the Tasmanian devil (Pye et al., 2016).

Exploring the immune escape mechanisms of DFT1 has been central to understanding how these foreign tumour cells establish in individuals without eliciting an alloresponse. The down-regulation of the major histocompatibility complex class I molecule (MHC-I) on the tumour cell surface is recognized as a principle mechanism for DFT1's immune evasion (Siddle et al., 2013). The immune escape mechanisms of DFT2 are yet to be identified.

The discovery of a second transmissible cancer affecting Tasmanian devils has raised many questions about the cancer itself, but also regarding the devil's susceptibility to such cancers. In light of recent evidence for immune recognition of DFT1 in both wild and immunised devils (Chapters 4, 5 and 6), one immediate concern was whether immune cross-recognition of DFT1 and DFT2 could occur. To explore this, serum samples from three cohorts of devils were tested

for IgG antibody binding to DFT2 cells. The first cohort consisted of wild devils with clinical signs of DFT1 or DFT2 or, in one case, both tumour types. This group had no previous demonstration of serum antibodies against either DFT1 or DFT2. The second cohort was a group of wild devils with demonstrated anti-DFT1 IgG antibodies (Chapter 4). The third cohort was a group of devils immunised with DFT1 preparations that had developed serum IgG antibodies against DFT1 (Chapters 5 and 6). In accordance with previous experiments on DFT1 cells, each serum sample was tested against un-manipulated, cultured DFT2 cells, and also against interferon gamma (IFN-γ) treated DFT2 cells. The expression of beta 2 microglobulin (β_2m), a component of the MHC-I molecule and thus an indication of surface MHC-I expression, was assessed for both untreated and IFN-γ treated DFT2 cells. The DFT2 antibody responses for each serum sample were compared to the responses against DFT1 cells.

7.2. Materials and methods

7.2.1. Cell culture

This study used two different DFT2 cell lines: Snug/TD500, collected and established in culture in October 2014; and TD549, collected and established in culture in December 2015. Tumour cells from the devils "Snug/TD500" and "TD549" were collected by fine needle aspirate from tumours during necropsies, which were carried out immediately after the devils were euthanased. The aspirates were collected with 21 G needles and 5 ml syringes and the cells placed directly into 5 ml of transport medium. Within a few hours of collection, the tumour cells were centrifuged (500 g for 5 minutes), resuspended in 5 ml Amniomax cell culture medium, placed in T25 flasks and incubated at 35 °C with 5% CO_2 in air.

Once the tumour cells were established in culture and proliferating, a sample was sent to the Animal Health Laboratory, Mount Pleasant for cytogenetic analysis as described in Chapter 2.2.10. Both Snug/TD500 and TD549 were confirmed as DFT2 by karyotype. The cell cultures were maintained in "super complete" medium i.e. complete medium with 20% Amniomax.

7.2.2. Serum samples from Tasmanian devils

Serum samples from 18 devils were tested for specific antibody binding to the two DFT2 cell lines using flow cytometry. Devils were divided into three groups according to their histories (Table 7.1):

Group 1. (n=6) Wild devils with clinical signs and histological confirmation of DFT1 and/or DFT2. Serum was collected at only one time point for each of these devils.
Group 2. (n=6) Wild devils from West Pencil Pine in north west Tasmania that had previously demonstrated antibody responses against MHC-I^{+ve} DFT1 cells. For each of these devils there were pre and post seroconversion serum samples that were tested.
Group 3. (n=6) Devils immunised with MHC-I^{+ve} DFT1 cell preparations that developed antibody responses against both MHC-I^{-ve} and MHC-I^{+ve} DFT1 cells. The pre-immune and post immunisation serum samples for each of these devils were tested.

Table 7.1. Details of devils and serum samples.

Group 1. Wild devils with DFT1 and/or DFT2					
Name/ ID	Estimated YOB	Sex	Location	DFT1 or DFT2 or both	Serum date
TD550	2014	M	Snug	DFT1 and DFT2	Jan 2016
TD549	2012	M	Woodbridge	DFT2	Dec 2015
TD467	2011	M	Cygnet	DFT2	March 2014
TD500	2011	M	Snug	DFT2	Oct 2014
Savuti	2011	F	WPP*	DFT1	Nov 2013
Winky	2013	M	Bronte	DFT1	May 2015
Group 2. Wild devils from WPP* with antibodies against MHC-I^{+ve} DFT1 cells					
Name	Chapter 4 ID	Estimated YOB	Sex	Pre seroconversion serum date	Date of seroconversion
Gengibre	TD1	2006	F	Nov 2009	Nov 2011
Esquivel	TD2	2006	F	May 2009	Nov 2011
Nairobi	TD3	2009	F	Feb 2011	Feb 2014
Trujillo	TD4	2008	M	July 2011	May 2014
Oryx	TD5	2008	F	Nov 2010	May 2011
Falestinya	TD6	2010	M	Nov 2013 (DFT1+)	Feb 2014
Group 3. Devils immunised in trials (Chapters 5 and 6) with subsequent antibody development against both MHC-I^{-ve} and MHC-I^{+ve} DFT1 cells					
Name	Referred to in chapter 5 or 6	YOB	Sex	Pre-immmune serum date	Post immunisation serum date
Badger	5	2008	F	28/03/13	03/07/13
Stinky	5	2007	F	12/02/14	13/05/15
Tip	5	2008	M	12/02/14	13/04/15
Lysa	6	2014	F	09/04/15	17/09/15
Chilli	6	2014	M	04/05/15	15/09/15
Andrea	6	2011	F	12/02/15	17/09/15

* WPP = West Pencil Pine, north west Tasmania

7.2.3. Flow cytometry

Indirect immunofluorescence and flow cytometry to measure serum anti-DFT2 IgG antibody levels was performed on all the serum samples against both Snug/TD500 and TD549 cell lines. Serum samples were tested with the untreated cell lines and also with IFN-γ treated cells. MHC-I surface expression was assessed on both untreated and treated cells with anti-beta 2 microglobulin (β_2m) with flow cytometry. See Chapter 2 for MHC-I up-regulation (2.2.5) and flow cytometry (2.2.7) methods. The median fluorescence intensity ratio (MFIR) was used to classify the antibody responses. The MFIR is the median fluorescence intensity (MFI) of DFTD cells labelled with immune serum divided by the MFI of DFTD cells labelled with pre-immune serum. The MFIR for the WPP and immunised devils was calculated by dividing the MFI at time of seroconversion by the "negative"/pre-immune serum MFI. This accounted for background serum IgG present, as well as inevitable variations between flow cytometry experiments. There was no such "constant" for the DFTD-affected wild devils in group 1 since only one serum sample was available for each. Therefore, the serum sample of one of these devils (TD550) was used as the constant i.e. the MFI of each of the serum samples of the devils in this cohort were divided by the MFI of TD550's serum sample (therefore TD550's MFIR = 1).

7.2.4. Statistical analyses

Student's paired t tests were performed to compare antibody responses for each group of devils against untreated and IFN-γ treated DFT1 and DFT2 cells.

7. 3. Results

7.3.1. Surface expression of MHC-I by DFT2 cells

Cell surface expression of MHC-I was assessed by staining DFT2 cells with β_2m antibody both before and after the cells were incubated for 24 hours with IFN-γ. The results were compared to DFT1 cells. The IFN-γ treated DFT1 cells were positive for β_2m, whereas the untreated cells were negative (Fig 7.1a). DFT2 cells showed some positive staining for β_2m prior to treatment but this was dramatically increased after the IFN-γ incubation (Fig 7.1b).

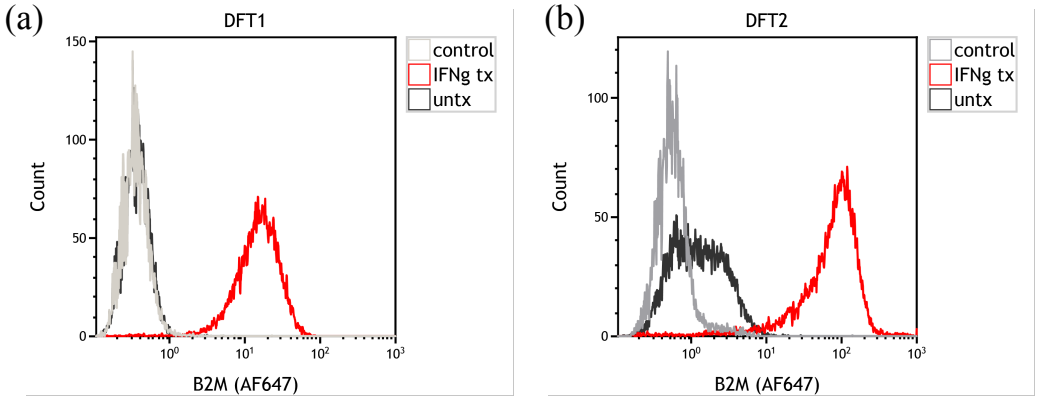


Figure 7.1. MHC-I expression, as indicated by β_2m surface staining, of untreated (untx) and IFN-γ treated (IFNg tx) (a) DFT1 cells and (b) DFT2 cells. The control samples are without the β_2m antibody.

7.3.2. Serum IgG antibody responses against untreated and IFN-γ treated DFT2 cells

Serum samples from 18 devils, divided into three groups according to their histories, were tested for specific antibody binding to DFT2 cells with flow cytometry. The samples described in 7.2.2 were assessed for IgG antibody binding to untreated DFT2 cells and also to IFN-γ treated DFT2 cells. The results reported are for serum samples tested on the Snug/TD500 cell line (representative of both DFT2 cell lines tested). The antibody responses to DFT2 for each serum sample were then compared to those measured on DFT1 cells. All antibody responses are reported as mean fluorescence intensity ratio (MFIR).

Group 1. Wild devils with DFTD

Serum samples from six wild devils (two with DFT1, three with DFT2, and one devil with both DFT1 and DFT2) were assessed for serum antibodies to cells of both DFT1 and DFT2. None of these devils had serum antibodies to untreated or IFN-γ treated DFT1 or DFT2 cells. Responses are shown for the devils with DFT2 against DFT2 cells in Figure 7.2.

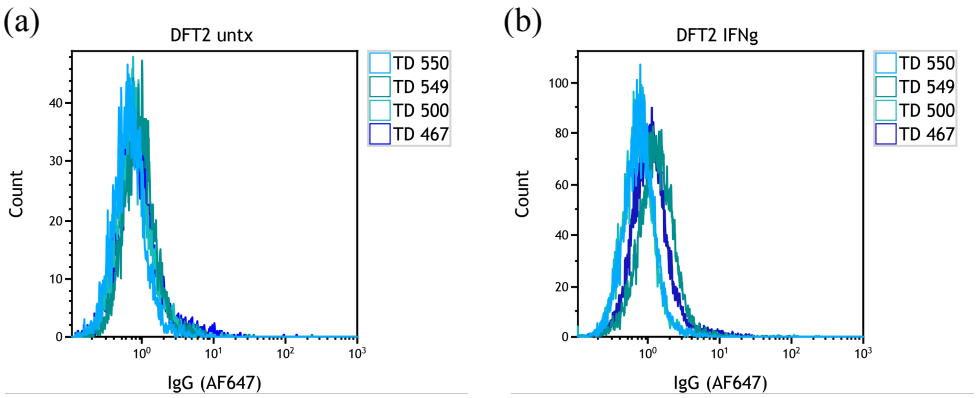


Figure 7.2. Group 1. IgG antibody responses of the three wild devils with DFT2 (TD467, TD500, TD549), and TD550 which had DFT1 and DFT2 against (a) untreated (untx) DFT2 cells, (b) IFN-γ treated DFT2 cells.

Group 2. Wild devils with antibodies against MHC-I^{+ve} DFT1

In Chapter 4 it was shown that six wild devils had antibody responses to IFN-γ treated (i.e. MHC-I^{+ve}) DFT1 cells. These devils didn't have antibody responses against MHC-I^{-ve} DFT1 cells. Serum samples from these devils were tested with untreated and IFN-γ treated DFT2 cells. For the untreated DFT2 cells, four devils (7.3a-d) had no antibody response in keeping with their DFT1 responses. However, two devils (Fig 7.3e,f) showed antibody responses to untreated DFT2 cells. All these devils had antibody that could bind to IFN-γ treated DFT2 cells (Figure 7.4).

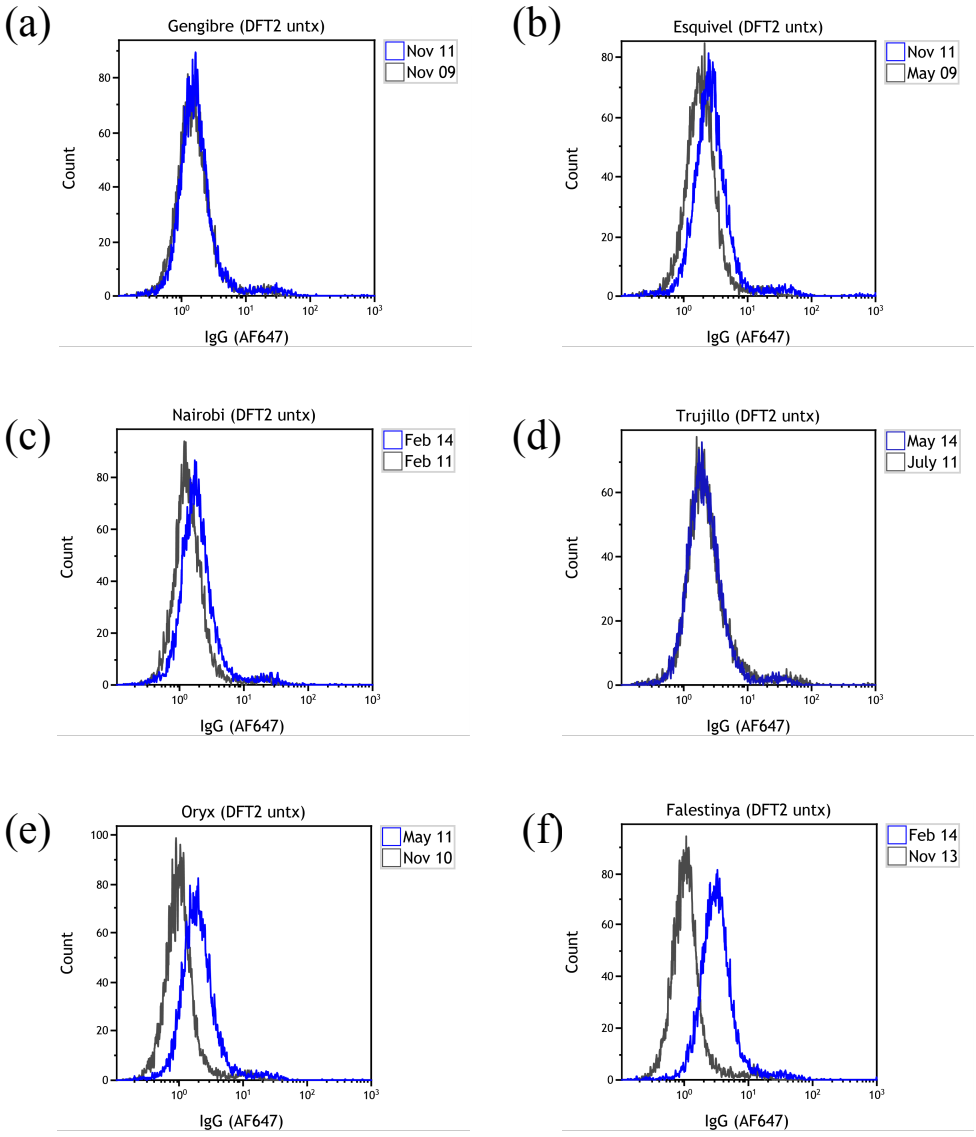


Figure 7.3. Group 2. IgG antibody responses against untreated (untx) DFT2 cells found in six wild devils with antibodies against MHC^{+ve} DFT1 cells. Devils shown in (a) to (d) had no antibody response whereas those in (e) and (f) had antibody recognizing untreated DFT2 cells.

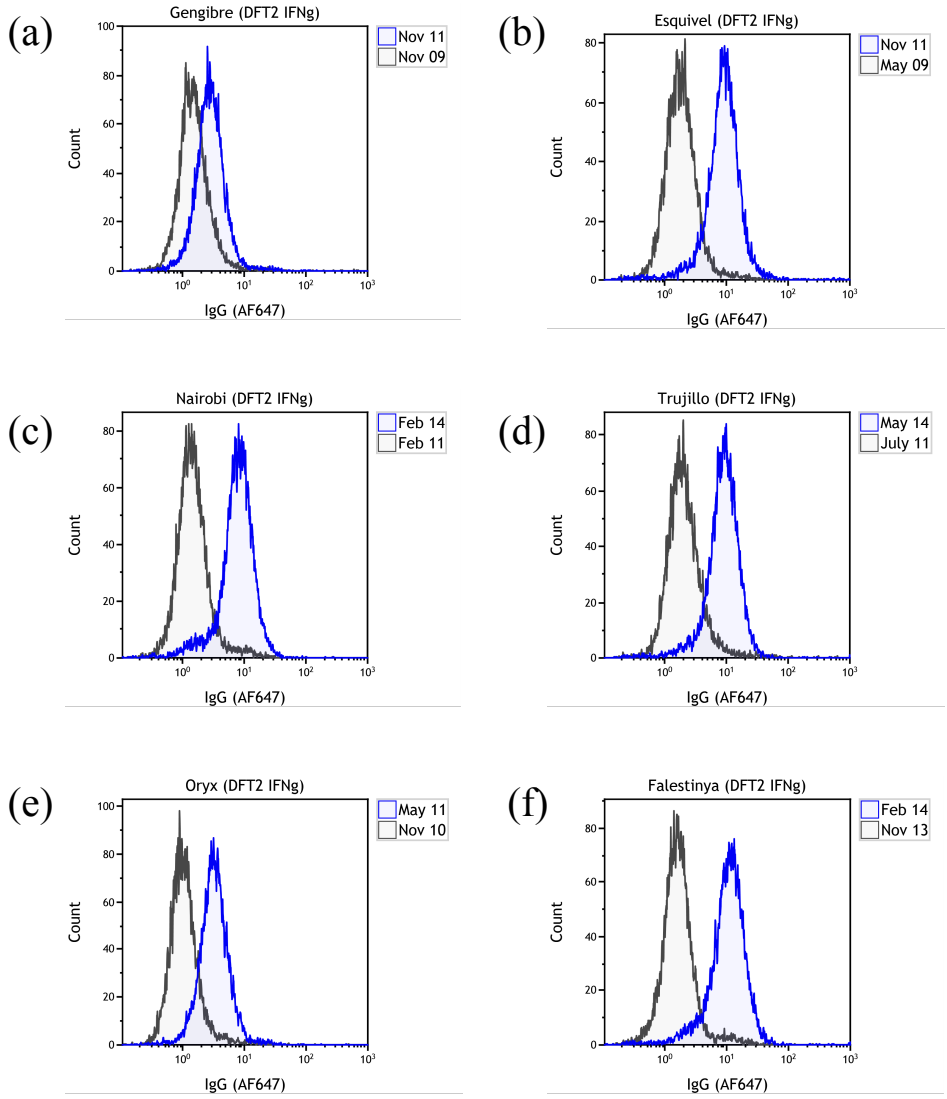


Figure 7.4. Group 2. IgG antibody responses against IFN-γ treated DFT2 cells found in six wild devils with antibodies against MHC-I^{+ve} DFT1 cells. All devils shown in (a) to (f) had antibody recognizing IFN-γ treated DFT2 cells.

Group 3. Captive devils immunised with MHC-I^{+ve} DFT1 cell preparations

DFTD (DFT1) immunisation trials have resulted in devils producing antibodies able to bind to both MHC-I^{-ve} and MHC-I^{+ve} DFT1 cells. Serum samples from six devils from the trials described in Chapters 5 and 6 were tested to assess whether they had similar antibody responses to DFT2. There were three devils selected from the pilot trials described in Chapter 5, and three selected from the field trial described in Chapter 6. All the devils except for one showed antibody responses to untreated DFT2 cells (Fig 7.5). All the devils had antibody responses to IFN-γ treated DFT2 cells (Fig 7.6).

Comparison of antibody responses to DFT1 and DFT2

The differences between the antibody responses to DFT1 and DFT2 cell lines were then analysed with Student's paired t tests for each group of devils i.e. wild devils with DFTD; wild devils with previous demonstration of anti-DFT1 responses; and immunised devils (Figure 7.7). The only significant difference between responses for either tumour type for any of the three groups of devils was found in the wild devils that had demonstrated antibodies to MHC-I^{+ve} DFT1 cells. While they all showed robust antibody responses to IFN-γ-treated DFT2 cells, the average MFIR was 4.6 compared to an average MFIR of 7 for antibodies against MHC-I^{+ve} DFT1 cells.

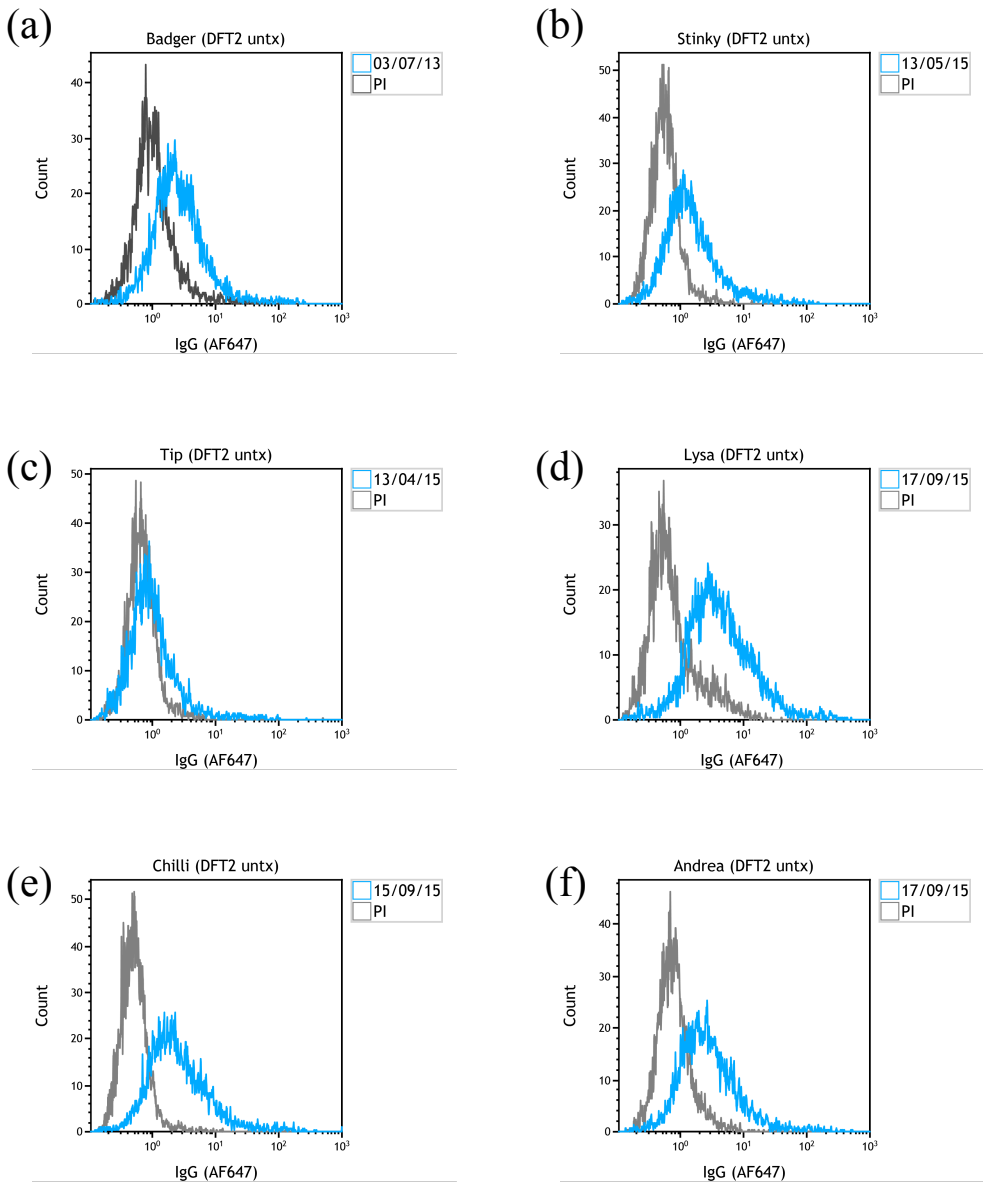


Figure 7.5. Group 3. IgG antibody responses to untreated (untx) DFT2 cells found in six devils immunised against DFT1. Only the devil in (c) failed to show an antibody response to untreated DFT2 cells. This devil had a low antibody response against untreated DFT1 cells (Chapter 5).

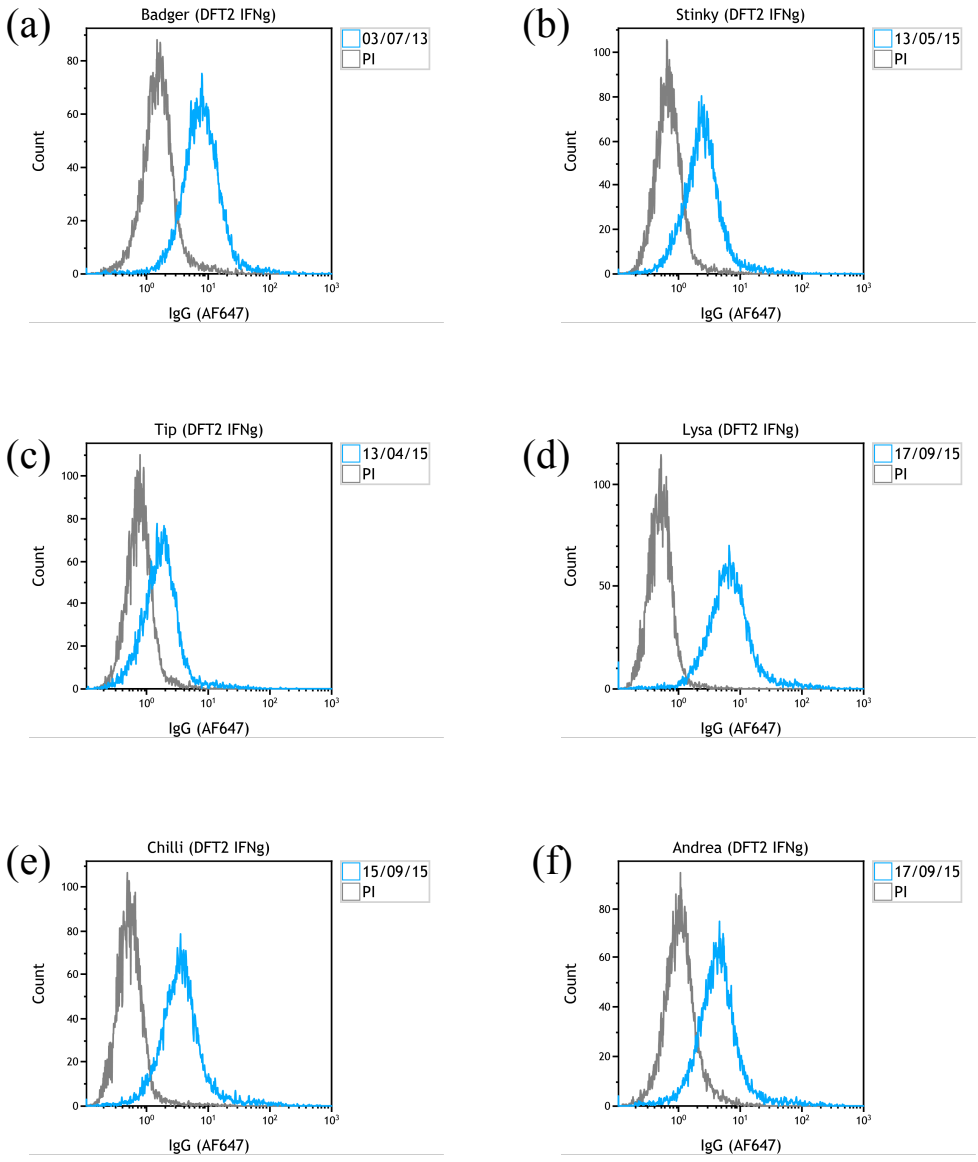


Figure 7.6. Group 3. IgG antibody responses to IFN-γ treated DFT2 cells found in six devils immunised against DFT1. All devils shown in (a) to (f) had antibody recognizing the treated DFT2 cells.

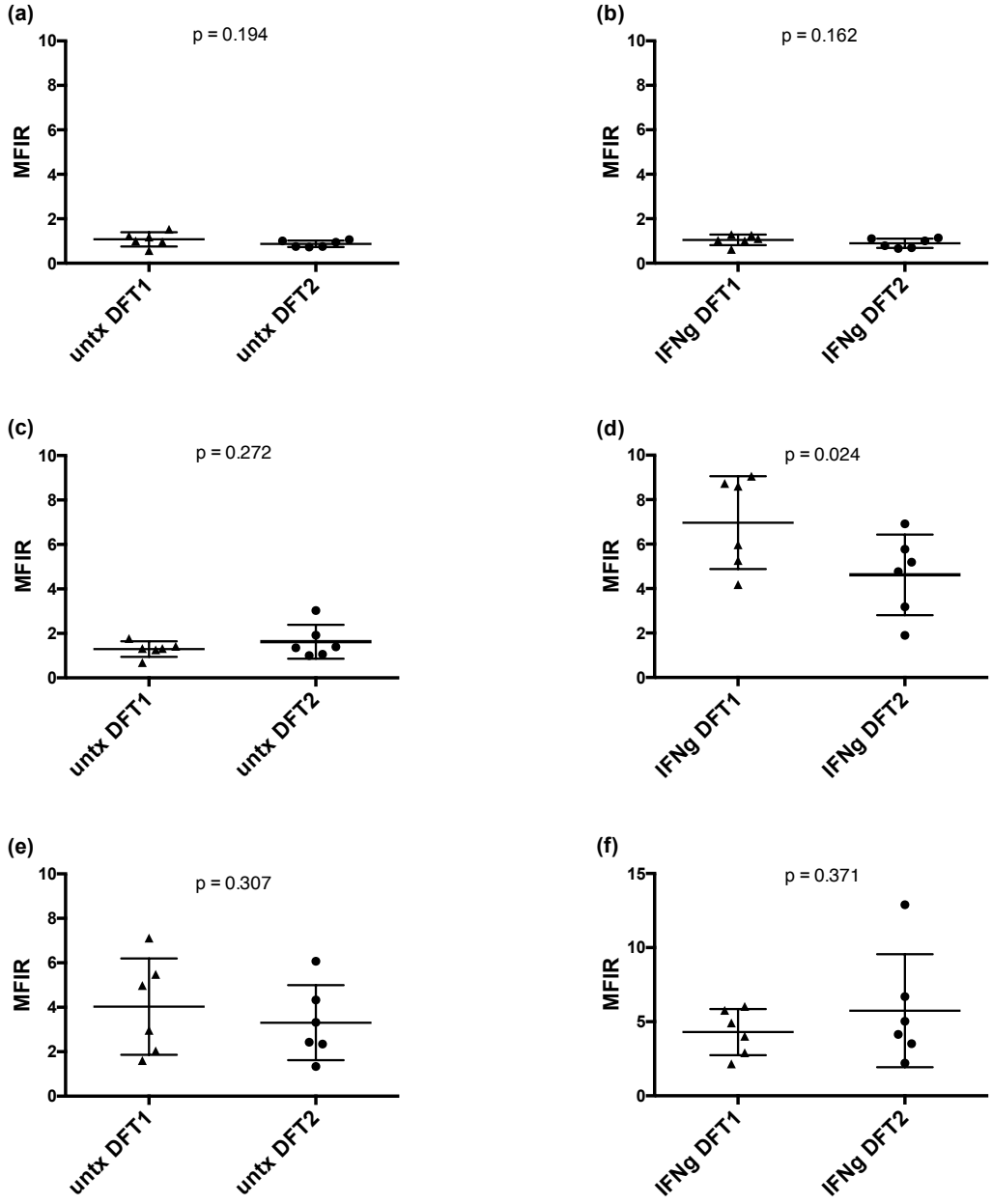


Figure 7.7. Summary of responses of all devils to untreated (untx) and IFN-γ treated DFT1 and DFT2 cells. (a), (c) and (e) are untreated cells; (b), (d) and (f) are treated cells; (a) and (b) are wild devils with DFTD; (c) and (d) are wild devils with previously demonstrated immune responses against MHC-I^{+ve} DFT1 cells; (e) and (f) are immunised devils. P values are the results of Student's paired t tests.

The wild devils with DFTD (a,b) had no antibody responses to untreated or IFN-γ treated DFT1 or DFT2 cells. The wild devils with antibody responses to treated DFT1 cells (c, d) had antibodies to treated DFT2 cells. These devils on average had no response to untreated DFT1 cells, and a low but not significantly different response to untreated DFT2 cells. The immunised devils (e, f) overall had antibodies to both untreated and treated DFT1 and DFT2 cells. MFIR = median fluorescence intensity ratio, as measure of antibody response.

7.3.3. Statistics analysis

Table 7.2. The t and p values for Student's paired t tests (2 tailed) comparing three different groups of devils' antibody responses to untreated (untx) and IFN-γ treated DFTD cells.

	Group 1 Wild devils with DFTD		Group 2 Wild devils with antibodies to DFT1		Group 3 Immunised devils	
Cell type	t	p	t	p	t	p
untx DFTD cells	1.501	0.194	1.234	0.272	1.138	0.307
IFN-γ treated DFTD cells	1.638	0.162	3.197	0.024	0.983	0.371

7.4. Discussion

Until recently it had been thought that transmissible cancers are extremely rare. Prior to 2015, only two such cancers had ever been observed in nature, DFTD in Tasmanian devils and CTVT in domestic dogs. That number doubled in 2015 with the discovery that leukaemia in soft shell clams is transmitted between individuals, and that a second transmissible tumour is affecting the devil. In 2016, a further four transmissible cancers were reported in shell fish including one cancer that had crossed species (Metzger et al., 2016). These authors concluded that transmissible cancers may be more common in nature than was first appreciated. Although we found the two cancers affecting the devil to be very different at the molecular level, DFT1 and DFT2 are grossly similar, with both causing tumours in the oral mucosa and/or facial skin (Pye et al., 2016). This study aimed to determine if there is common antigenicity between the tumour cell types.

None of the six wild devils in Group 1 with clinical signs of DFTD (DFT1, DFT2, or both) had serum antibody against either tumour type. This was not unexpected given that the previous study described in Chapter 4 found anti-DFT1 immune responses in only 10% of wild devils from a particular population.

The six wild devils with anti-DFT1 responses in Group 2 either had histories of DFT1 tumour regression, or tumours with evidence of immune cell infiltration or MHC-I expression. All six

of these devils showed detectable antibody binding to IFN-γ treated DFT2 cells. However, this was not as high as that against treated DFT1 cells. This suggests either these devils have a lower antibody titre for DFT2, or there are fewer specific target antigens on DFT2 cells. None of these devils had shown a significant response to MHC-I^{-ve} DFT1 cells but two of them seemed to have antibodies to untreated DFT2 cells. These were at lower levels than their antibodies to IFN-γ treated DFT2 cells, and didn't contribute to an overall significant difference between the responses to untreated DFT1 or DFT2 cells in this cohort of devils. It is interesting to note that neither of the devils with antibodies to untreated DFT2 cells had a history of observed tumour regression. This is in contrast to the other four devils. It is possible these two devils had autoantibodies recognising antigens on the untreated DFT2 cell surface, or the degree of MHC expression on these untreated DFT2 cells is responsible for the antibody response. However, further analysis of DFT2 cells with serum from a larger number of wild devils (as was carried out in Chapter 4) should be performed before drawing conclusions about these differing responses.

Devils immunised with MHC-I^{+ve} DFT1 cell preparations developed antibodies against both MHC-I^{-ve} and MHC-I^{+ve} DFT1 cells (Chapters 5 and 6). These devils show similar antibody responses against both untreated and IFN-γ treated DFT2 cells. The only immunised devil not to show serum antibodies for untreated DFT2 cells was "Tip". This devil underwent tumour regression following immunotherapy, and had a low antibody response against untreated DFT1 cells as shown in Chapter 5. He did however have antibodies recognizing both IFN-γ treated DFT1 and DFT2 cells. In this respect his response was similar to the four wild devils that had histories of DFT1 tumour regression and antibodies that only recognized IFN-γ treated cells. The overall results from this cohort of devils suggest that immunising devils with DFT1 cell preparations will produce immune responses against both tumour types. Assuming the antibody responses provide some correlation with protectiveness against DFTD, this bodes well for a single vaccine protecting against both tumours. However, it might be more efficacious to include DFT2 in the vaccination protocol to ensure coverage.

The MHC-I expression of DFT2 *in situ* is yet to be clarified but is already proving an intriguing aspect of DFT2 research. It is well established that DFT1 cells, at least in culture, have down-regulated surface expression of MHC-I (Siddle et al., 2013). Experiments have shown that at least a proportion of untreated DFT2 cells show some positive staining for β_2m when compared

to untreated DFT1 cells. This suggests some surface expression of MHC-I in the un-manipulated DFT2 cells, at least in culture. As occurs with DFT1 cells, this surface MHC-I expression is dramatically elevated when the DFT2 cells are treated with IFN-γ. It has been confirmed that DFT2 has a different MHC-I genotype from DFT1 (Pye et al., 2016).

It would be curious if foreign cells expressing surface MHC-I do not elicit an alloresponse in devils. Despite low genetic diversity, devils have demonstrated immune rejection of allografts (Kreiss et al., 2011). One possible explanation for the lack of response against the MHC-I molecules expressed on DFT2 cells is that these are non-classical MHC-I (Hannah Siddle, pers. comms 2015). Non-classical MHC-I molecules can be over-expressed by malignant cells and inhibit the cytolytic activity of various effector cells (Kochan et al., 2013) thus providing a mechanism of immune escape for neoplastic cells. The particular responses of the wild devils with DFTD seroconversion suggests that classical MHC-I expression is a target for naturally occurring immune recognition of DFTD.

The serum antibody results reported here suggest there is common antigenicity between DFT1 and DFT2 cells. Further analysis of the serum antibodies using western blotting and 2D gel electrophoresis may aid in identifying the common antigens between the two tumours. Antibodies from the seroconverted wild devils may also help determine the significance of MHC-I in the immune responses against DFTD.

Chapter 8

Final discussion

Chapter 8. Final discussion

8.1. The Tasmanian devil, DFTD and vaccine development

The Tasmanian devil (*Sarcophilus harrisii*) has been listed as an endangered species due to an aggressive, transmissible and fatal cancer known as devil facial tumour disease (DFTD) (Hawkins et al., 2008). The devil is the world's largest extant carnivorous marsupial and unique to Australia's island state of Tasmania. DFTD is a contagious cancer, passed between devils by biting. Transmissible cancers are rare events whereby the tumour cells are the sole aetiological agent. They require a means to infect new hosts, as well mechanisms to evade the host's immune system. Until 2015, the only other transmissible cancer known to exist was canine transmissible venereal tumour (CTVT), an ancient tumour affecting domestic dogs (Murgia et al., 2006). Another six transmissible cancers have since been reported, with five occurring in shellfish, and one in Tasmanian devils (Metzger et al., 2015, Metzger et al., 2016, Pye et al., 2016).

The extinction in the wild of the Tasmanian devil would have profound impacts. It is an internationally recognised species with an iconic status that inhabits a unique ecological niche. As devil populations decline this niche is at risk of being filled by feral cats and potentially foxes, the consequences of which would prove disastrous for native species (Hollings et al., 2014, Hawkins et al., 2006). Although no local devil population extinctions have been recorded, the devil has been described as functionally extinct in certain parts of the state (Hollings et al., 2015). There is evidence suggesting that DFTD is influencing genetic selection (Epstein et al., 2016), but there is no sign yet that the effect of DFTD is abating. Consequently, human intervention in the form of carefully thought out management plans may be necessary to restore a genetically diverse wild devil population. This would include the development of a protective vaccine against DFTD.

Research towards the goal of vaccine development has been in progress since 2006. Clearly it is a long term and challenging commitment. The aims of this thesis were formed within the context of DFTD vaccine development, and studies were carried out on wild and captive devil populations. The wild devil population provides great scope for exploring DFTD, the devil's immune system and how the two interact. Such knowledge, in particular how it relates to natural conditions, is essential for vaccine development. Part of this thesis made use of field

studies on wild devils to inform on the "normal" devil immune system with respect to T lymphocytes and immunoglobulins, and how these are affected by DFTD. This included identifying specific immune responses against DFTD in the wild devils.

The captive devil population has proven extremely useful for vaccine research, particularly in respect to the implementation of immunisation trials. The advantages of working with captive devils include their assured non-exposure to DFTD due to strict quarantine conditions, and the guaranteed follow up of individual devils. Previous DFTD immunisation trials on small numbers of captive devils, usually two or three individuals per trial, have yielded encouraging preliminary results. In particular, they have shown that devils are capable of mounting both humoral and cell mediated immune responses against DFTD (Kreiss et al., 2015). The limited number of individual devils available for trials has meant uncertainty regarding how the wider devil population is represented. Nonetheless notable results in this thesis arose from the small immunisation trials described in Chapter 5. There was evidence of an immune response consistently generated against MHC-I^{+ve} DFTD cells. More importantly, there was convincing evidence for immune mediated tumour regression in immunised devils with DFTD following immunotherapy. These results serendipitously coincided with the initial stages of the Save the Tasmanian Devil Program's (STDP) "wild devil recovery" project. The devils earmarked for the first wild release were made available for immunisation trials prior to their release. This provided the opportunity to address sample size restrictions for the first time. Consequently, this thesis included the implementation of a field immunisation trial.

Lastly, the discovery of a second transmissible cancer affecting Tasmanian devils has raised many questions regarding transmissible cancers: their prevalence, their immune escape mechanisms, and the susceptibility of devils to such cancers. An immediate question for those involved in DFTD vaccine research was whether immune cross-recognition of the tumours could occur. The final aim of this thesis was to identify antibody responses against DFT2 in both wild and immunised captive devils.

8.2. Effects of DFTD on the immune response of the Tasmanian devil

In general, malignant cancers compromise the immune response of the host (Whiteside, 2006, Whiteside, 2010). This is particularly relevant at the tumour site whereby the tumour

microenvironment is characterized by a variety of immunosuppressive factors e.g. TGF-ß and IL10; and suppressor cell populations e.g. T regulatory cells (Whiteside, 2006). At a systemic level, the circulating T lymphocytes of patients with malignancies have been shown to be functionally compromised (Hellstrom et al., 2001). Since T cell function is critical for anti-tumour activity, it follows that a dysfunctional population facilitates tumour progression.

DFTD is an aggressive malignant cancer, with death usually resulting within 6 to 12 months of the tumour's first appearance (Hawkins et al., 2006). It is therefore logical to assume that it impacts on the devil's immune system, including the T lymphocyte population. Knowledge of the make-up and activity of T cell populations in healthy and diseased devils is necessary for understanding the interaction between the devil's immune system and DFTD. Advances in this area continue within the confines of limited reagents for investigation. DFTD has previously been shown not to affect lymphocyte proliferation *in vitro* (Kreiss et al., 2008). The distribution and abundance of CD4+ and CD8+ T cells in lymphoid tissue of heathy devils and devils with DFTD has also been documented (Howson et al., 2014). One of the aims of this thesis was to address the T lymphocyte populations in peripheral blood of wild devils, and to ascertain the effect of DFTD on these circulating cells. This would indicate whether immunocompromise is a consequence of DFTD.

A primary finding was that DFTD is associated with reduced percentages of CD4+ and CD8+ lymphocytes in the peripheral blood. This was accompanied by an increased percentage of CD4-CD8- lymphocytes. The identification and activity of these 'double-negative' T cells remains undetermined. It was hypothesized in Chapter 3 that immunosuppressive cytokines released by the tumour cells might cause reduced expression of the CD4 and CD8 molecules. Peripheral T lymphocytes from patients with advanced cancer have demonstrated low expression of CD3, CD4 and CD8 molecules in comparison to lymphocytes from healthy donors (Hellstrom et al., 2001). Proliferation and cytokine release was diminished in these cancer patients' lymphocytes. The function of the 'double negative' T lymphocytes in devils with DFTD could also be compromised as CD4 and CD8 are important molecules involved in antigen presentation and T cell activation. A reduced density of these molecules would impair T cell activation and result in reduced T cell immunity, and conceivably promote the progression of DFTD.

The novel method described in this thesis to identify and quantify the peripheral blood CD4+ and CD8+ T lymphocytes in devils was particularly useful for the field situation. Preservation of the blood clots was quick, requiring no special equipment and only buffered formalin for fixation. Storage and transport of the fixed clots was at ambient temperature, and there was no time limit in which to analyse the samples. While the tests used to validate the method showed it was useful for quantifying the average percentages of lymphocytes for devils in a particular cohort, it wasn't accurate enough to confidently quantify the percentages in an individual. Further validation of the method will be possible with the availability of more reagents, in particular an anti-devil CD8 antibody for use in fresh blood. Despite the limitations of the method, there was a clear finding that DFTD reduces the normal T lymphocyte population.

Most of the literature discusses immunocompromise of the cancer patient in terms of reduced lymphocyte counts and lymphocyte function (Whiteside, 2006, Kim et al., 2007). The humoral immune response is examined more specifically with respect to antibodies against tumour associated antigens (Reuschenbach et al., 2009, Dudas et al., 2010). Detection of antibodies against DFTD in wild devils was an important part of this thesis, but the effect of DFTD on total serum IgG and IgM levels was also explored. The increased serum levels of IgM and IgG noted in this study could be explained by increased exposure to pathogens in general. Both IgM and IgG were at higher levels in healthy adults than healthy juveniles. This is typical of other species and considered a normal consequence of maturity (Schreiber et al., 1992). DFTD was found to affect the IgM:IgG ratio in a study that examined serum mRNA (Ujvari et al., 2016). However, the study described in this thesis found differences for IgG and the IgM:IgG ratio between healthy and diseased devils only in the juvenile cohort. This points to the complexity of factors, including environment and age, involved in the effect of cancer on various immune parameters.

The immunoglobulin results reported in this thesis suggest the devils with DFTD still have a functional humoral immune response. The T lymphocyte populations however, are adversely affected by DFTD in line with the effects of malignant cancers on other species. This has implications for immunotherapy which requires a functional immune system to be effective. However, the effects of cancer on T cells are not necessarily irreversible. Restoration of lymphocyte function as measured by proliferation and cytokine release has been demonstrated *in vitro* with lymphocytes collected from patients with malignancies (Hellstrom et al., 2001). In addition, cytokine therapy has restored normal signaling in CD8+ T lymphocytes of patients

with malignant melanoma (Whiteside, 2006). Recognizing how the immune system is altered in devils naturally infected with DFTD informs on which responses are influenced by DFTD infection and, by extension, which pathways, for example cytokine release, might be important for DFTD rejection.

8.3. Immune responses against DFTD, and the role of MHC-I expression

The demonstration of immune responses against DFTD in wild devils described in Chapter 4 was a major finding of this thesis. It provided the first evidence that the devil's immune system can respond to DFTD and that death is not the inevitable outcome of DFTD infection. DFTD antibodies were present in all six of the wild devils with a response. A cell mediated immune response in the form of CD3+ and MHC-II+ infiltrating cells in tumour biopsies was evident in two devils. The tumour cells from a third devil showed MHC-I surface expression at the time of that devil's seroconversion.

The serum antibodies identified in these wild devils were against MHC-I^{+ve} DFTD cells, but not MHC-I^{-ve} cells. This suggests that MHC-I expression by DFTD cells is necessary to activate an anti-DFTD immune response. It is not clear what provides the initial trigger for MHC-I expression in the tumour cells. It is likely, however, that this trigger is a primary immune response that results in cytokine release by T helper cells and macrophages that are drawn to the tumour microenvironment. Since incubating DFTD cells with inflammatory cytokines *in vitro* results in the cells' dramatic up-regulation of MHC-I surface expression, it is reasonable to assume the same occurs *in vivo*. The cytokine release should have a cascade effect with increasing numbers of MHC-I^{+ve} DFTD cells attracting more cytotoxic lymphocytes resulting in killing of tumour cells and ultimately tumour stabilization or regression. The simultaneous seroconversion is explained by presentation of tumour peptides on the MHC-II molecules of antigen presenting cells to T helper cells, and the subsequent proliferation of B cells and antibody production. The antibodies could play a functional role in the anti-tumour response by antibody dependent cell mediated cytotoxicity (ADCC). This process has previously been reported in devils whereby the cytotoxicity against xenogeneic tumour cells demonstrated *in vitro* was attributed to natural killer (NK) cells acting in an antibody dependent manner (Brown et al., 2011).

The importance of the serum antibody response for DFTD rejection is further supported by

results from the immunised captive devils that underwent regression of their experimentally induced tumours, described in Chapter 5. These devils had an increase in their serum antibody levels to MHC-I^{+ve} DFTD cells following immunotherapy with live DFTD cells expressing MHC-I. This coincided with tumour regression. Even more distinctive was the seroconversion in the devil from the 2012 trial referred to in Chapter 5. This devil didn't have a measurable antibody response following immunisations. However, after tumour development and subsequent immunotherapy she developed a high level of serum antibodies to MHC-I^{+ve} DFTD cells, but no response to MHC-I^{-ve} cells. Once again, this coincided with complete tumour regression.

The most likely explanation for DFTD's avoidance of allorecognition is the lack of MHC-I expression on the tumour cells (Siddle and Kaufman, 2013). This results in a failure to activate MHC-I restricted cytotoxic T cells and tumour development is unhindered. Despite indirect evidence for devil NK cells (Brown et al., 2011), it is unknown why these cells fail to target the transmitted i.e. MHC-I^{-ve} DFTD cells. Anti-inflammatory cytokines such as TGF-ß inhibit the function of NK cells (Jakowlew, 2006) and unpublished data suggests these inhibitory cytokines are present in the DFTD microenvironment (Howson, 2011). This provides a potential explanation for the lack of NK cell activity. Likewise, some tumour cells don't express the requisite ligands for NK cell function (Cheng et al., 2013). The lack of NK cell activity might be reversed after an inflammatory response such as the one described above. The evidence from the wild and captive devils argues that if DFTD cells can express MHC-I, a response against the DFTD tumour cells will be activated.

A noteworthy parallel is that the role for MHC-I expression and antibody responses in canine transmissible venereal tumour (CTVT) rejection has been demonstrated. During the progressive phase of experimentally induced tumours, there is minimal surface expression of MHC-I on the CTVT cell surface. After 4 months, MHC-I expression is significantly increased and coincides with tumour regression. Both cell mediated and humoral responses occur during this regressive phase (Hsiao et al., 2008, Hsiao et al., 2002). Antibody dependent cell mediated cytotoxicity is enabled by the anti-CTVT IgG antibodies (Cohen, 1980). The development of allotypic antibodies in the serum of dogs following CTVT rejection suggests that tumour rejection involves the canine MHC system (Epstein and Bennett, 1974).

The evidence for the role of MHC-I expression for the development of immune responses against DFTD led to the next part of the study. This was an immunisation trial on 19 captive

devils prior to their wild release as the first stage of the STDP's "wild devil recovery" project. The use of DFTD cells expressing MHC-I as the antigenic basis for the immunisation trial described in Chapter 6 resulted in antibody responses against both MHC-I^{+ve} and MHC-I^{-ve} DFTD cells in the majority of devils. It is expected that cells expressing MHC-I are more immunogeneic than MHC-I^{-ve} cells due to presentation of cellular antigen by the MHC-I molecule. The adjuvants used in the immunisations are likely to have further enhanced the immune response to the cell preparations since multiple signalling pathways were targeted by the combination of TLR agonists and ISCOMATRIX™. MHC-I^{+ve} and MHC-I^{-ve} DFTD cells are likely to have antigens in common providing an explanation for the generation of antibody responses against both cell types in the immunised devils. Whether these responses translated to protection from a natural DFTD challenge currently remains untested. It may be possible to extend the trapping range beyond Narawntapu National Park in 2017 and locate some of the released devils that dispersed outside the park. This may provide an indication of the duration of immune responses and protectiveness of the immunisations.

8.4. Identification of DFTD epitopes for future vaccine development and immune response measurement

The above discussion highlights the apparent importance of MHC-I expression for immune recognition of DFTD. At this stage, however, the only certainty is that serum antibodies in devils with histories of tumour rejection are recognizing epitopes on DFTD cells that have been incubated with IFN-γ. Antibodies to MHC-I^{+ve} cells could be recognizing: the MHC-I molecule; the peptide presented by the MHC-I molecule; and/or other molecules that are also up-regulated by cytokine incubation but not necessarily associated with MHC-I. The other consideration is that immunised devils develop antibodies against MHC-I^{-ve} DFTD cells. Given these cells reflect the wild type tumour cells which lack MHC-I expression, it is logical that protection from a natural DFTD challenge also requires recognition of MHC-I^{-ve} DFTD cells. This theory however has not been properly tested.

It will take dedicated research to determine which DFTD epitopes are recognized by the serum antibodies from individual devils, and to what extent there is commonality. It is reasonable to assume that the serum antibodies identified in devils with tumour regression have functional relevance and/or prognostic value rather than simply denoting exposure or immunopathology.

Identification of DFTD epitopes recognized by serum antibodies from these devils has practical applications. It will allow for development of an epitope based vaccine as opposed to the whole cell preparation in current use. While advantages of whole cell vaccines were described in the literature review (Chapter 1.16.2), a vaccine comprised of target epitopes in combination with adjuvants could prove an effective approach. At the very least it would eliminate the risk of tumour inoculation as a result of the whole cell preparation.

The parallels between devil immunology / DFTD vaccine development and koala immunology/ chlamydia vaccine development were discussed in Chapter 1.16.4. The chlamydia vaccine trialled in koalas has relied on the chlamydia recombinant major outer membrane protein (rMOMP) as the antigen, rather than incorporating the whole chlamydia organism. The rMOMP, in combination with similar adjuvants to those used in DFTD immunisation trials, has resulted in seemingly protective humoral and cell mediated immune responses in koalas (Waugh et al., 2016). It remains to be seen whether an epitope-only DFTD vaccine will be effective in the devils.

The chlamydia rMOMP has also been instrumental in measuring the koalas' responses to the immunisations, and this is the other area to which identification of DFTD epitopes will contribute. Flow cytometry is the method currently used for antibody response measurement in devils. It identifies the presence of serum antibody to any epitope on the entire tumour cell surface. This method can't detect whether each devil has antibodies recognizing the same epitopes. The isolation of DFTD cell surface epitopes with immune serum from devils with histories of tumour regression should determine if these devils have anti-DFTD antibodies in common. Presumably these antibodies and the epitopes they recognize will be keys to DFTD immune recognition and rejection. As with koala vaccine research, these specific epitopes could then be used to assess antibody responses to immunisation trials. They could also be used to screen wild devil serum samples to assess the prevalence of naturally occurring immune responses. Cell mediated immune responses could also be tested with the epitopes e.g. by measuring lymphocyte proliferation in response to epitope stimulation.

8.5. DFT2 and final remarks

The final chapter of this thesis was based on DFT2, a second transmissible cancer affecting the devils that was discovered while this thesis was underway. While DFT1 and DFT2 are

indistinguishable at the macroscopic level, they have marked histological, cytogenetic and genetic differences, including distinct MHC-I genotypes (Pye et al., 2016). All analyses to date indicate that DFT2 is a transmissible cancer independent of DFT1. The confirmation of a second transmissible cancer affecting an already endangered species is troubling. However, DFT2 has been limited to the Channel peninsula in south east Tasmania with only 13 confirmed cases at the time of writing. It is currently open to speculation whether the Tasmanian devil is particularly susceptible to such events, whether there is an exogenous component, or whether it is pure chance that two independent transmissible cancers have appeared in the species within a relatively short time frame. The reporting of five transmissible cancers in clams (Metzger et al., 2015, Metzger et al., 2016) around the same time as the DFT2 discovery has prompted the question of whether transmissible cancers are more common in nature than has previously been assumed.

There remains much to find out about DFT2: its cellular origin; its immune escape mechanisms; and how it compares with DFT1. Despite the concerns DFT2 presents for the survival of the species, research into this new transmissible cancer will contribute to an understanding of immune escape and cancer evolution. On a more immediate and practical level, preliminary results presented here suggest that if the devils can mount an immune response against DFT1 this should translate as a response against DFT2. There was cross-recognition of DFT2 by all devils, wild and immunised, that had demonstrated antibodies against DFT1 cells. All of these devils had antibodies that recognized IFN-γ treated DFT2 cells, but only a proportion also had antibodies recognizing the unmanipulated DFT2 cells. DFT2's *in vitro* expression of MHC-I is distinct from that of DFT1. As discussed in Chapter 7, unmanipulated DFT2 cells show some level of MHC-I expression. This might be non-classical MHC-I which would help explain the lack of allorecognition of DFT2 tumours (Hannah Siddle, pers. comms 2015). It is clear that when DFT2 cells are incubated with the inflammatory cytokine IFN-γ, the MHC-I expression is dramatically increased. This provides further evidence for the role of MHC-I expression in immune recognition of DFTD.

The immune cross-recognition of DFT1 and DFT2 is encouraging especially in the light of recent evidence for immune responses against DFT1 in wild devils (Chapter 4), possible gene selection in reaction to DFT1 (Epstein et al., 2016), and promising results from DFT1 immunisation trials (Chapters 5 and 6). These are exciting developments and should be

acknowledged as such. Yet without evidence for the effects of DFTD abating, it is too soon for complacency. At an individual level, hundreds of devils continue to succumb to DFTD and die of starvation or metastatic disease. At a population scale, the future of the wild Tasmanian devil is not guaranteed. The challenges are complex, but the development of a protective vaccine against DFTD would prevent the suffering of countless individuals, help secure a genetically diverse wild devil population, and contribute to maintaining the unique Tasmanian ecosystem.

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Appendix 1

Protein electrophoresis

Table 1. Devil serum samples.

Sex	DFTD-		DFTD+	
	Devil name	Age (years)	Devil name	Age (years)
control (captive, male)	Prince	4	/	/
male	Wheery	1	Tylden	2
	Libano	2	Enfield	2
	Trujillo	6	Tiwanaku	2
female	Hoppity Hare	1	Maui	1
	Kialla	2	Savuti	2
	Chiricoca	4	Nungwi	3

The control devil was a captive devil, the others were wild devils from West Pencil Pine.

Table 2. Total protein, albumin and globulin ranges for healthy and DFTD+ devils.

DFTD status	Total protein g/L	Albumin g/L	Globulin g/L	A:G
DFTD-	59 - 68	32 - 40	24 - 32	1.09 - 1.50
DFTD+	57 - 67	25 - 35	29 - 36	0.86 - 1.10
control (DFTD-)	67	35	32	1.09

Table 3. Globulin fraction ranges for healthy and DFTD+ devils.

DFTD status	Alpha 1 g/L	Alpha 2 g/L	Beta 1 g/L	Beta 2 g/L	Gamma g/L
DFTD-	1.7 – 2.6	2.6 – 6.5	6.5 – 7.5	2 – 3.7	8.9 – 19.9
DFTD+	6.4 – 9.0	2.0 – 4.6	7.4 – 9.6	3.4 – 5.1	12.7 – 17.4
Control (DFTD-)	2.7	5.4	6.0	3.8	14.7

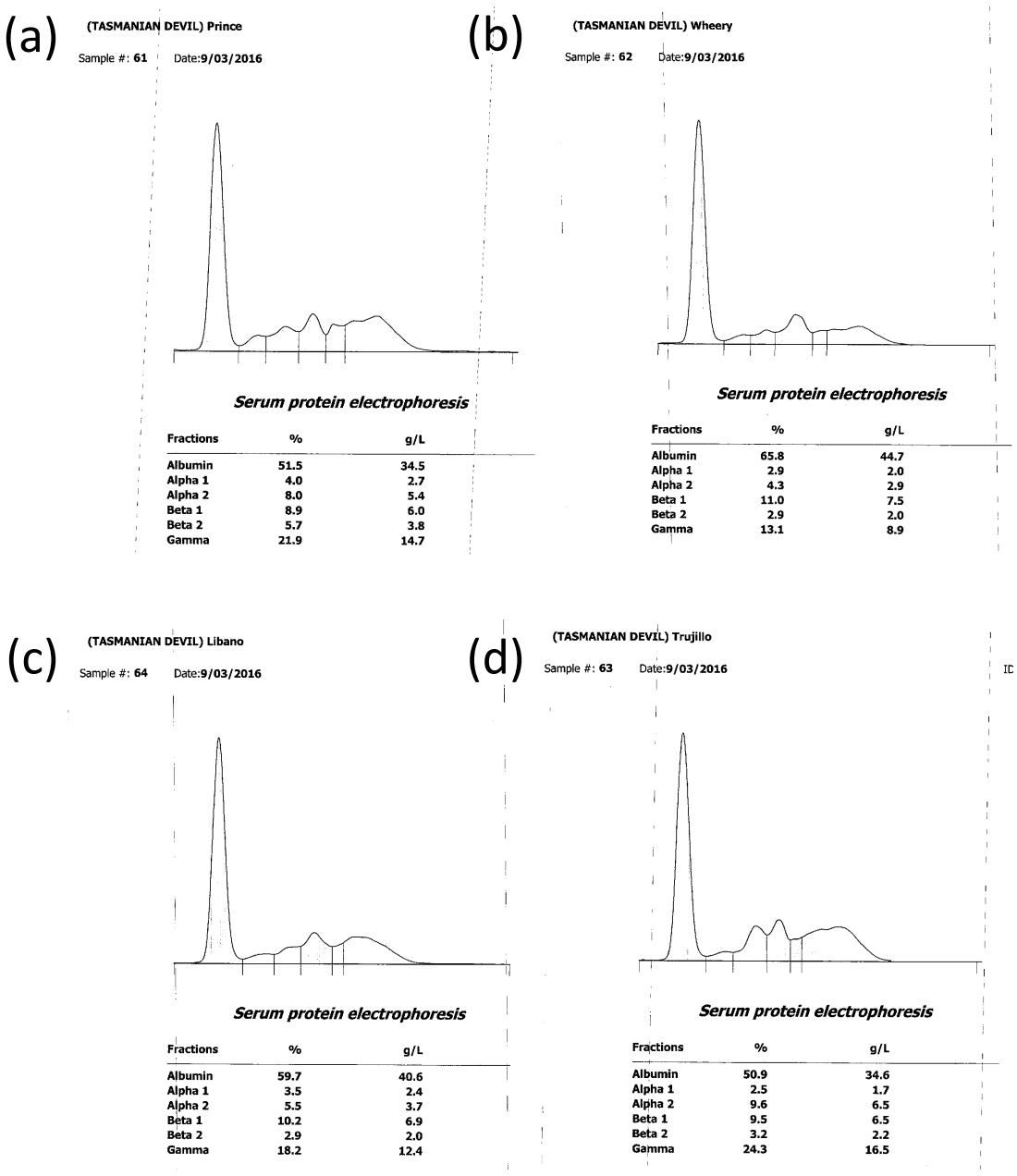


Figure 1. Electropherograms of healthy (DFTD-) male devils (a) control devil, 4 years old, (b) and (c) wild devils, both 2 years old, (d) wild devil, 6 years old.

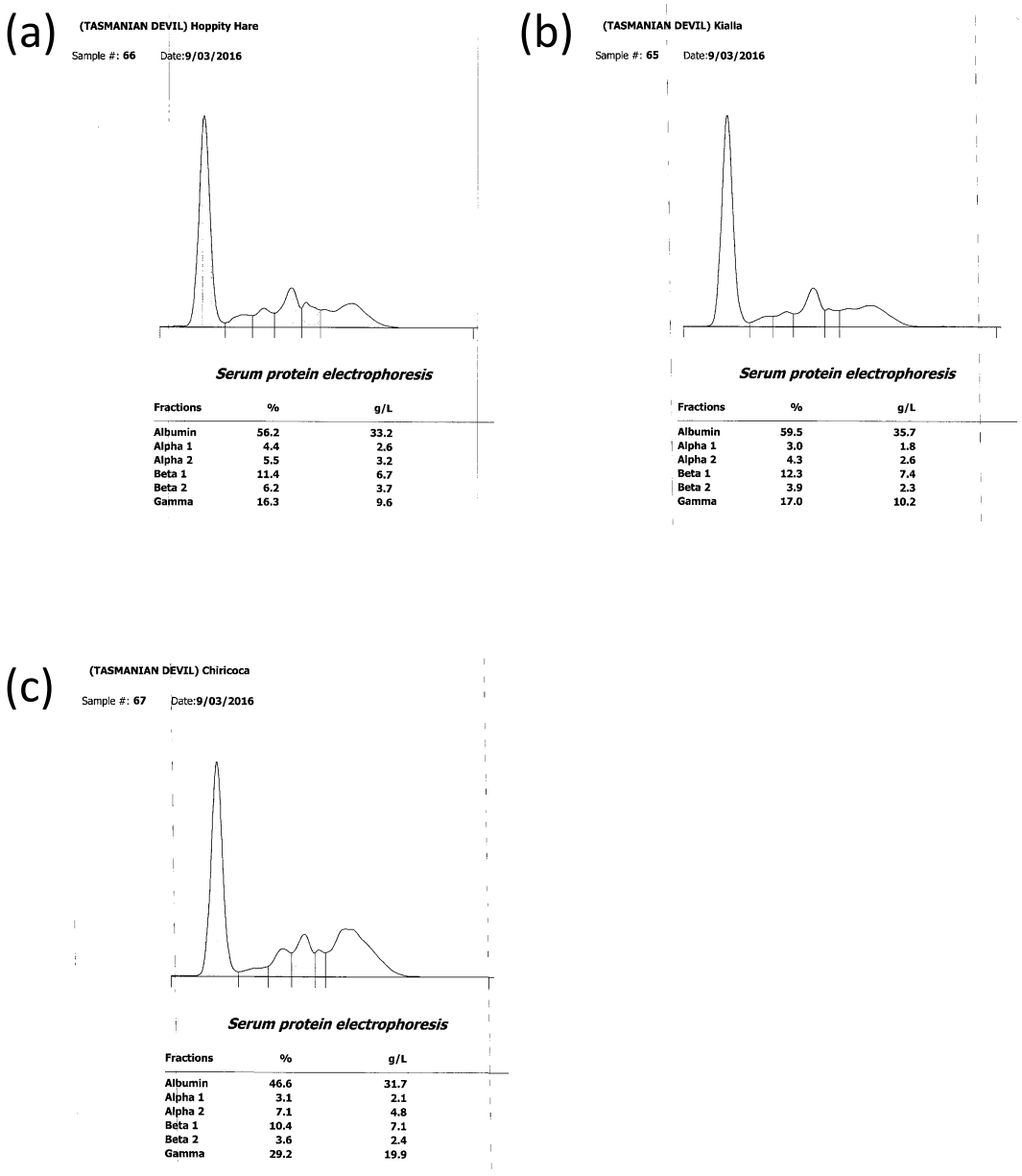


Figure 2. Electropherograms of healthy (DFTD-) wild female devils (a) 1 year old, (b) 2 years old, (c) 4 years old

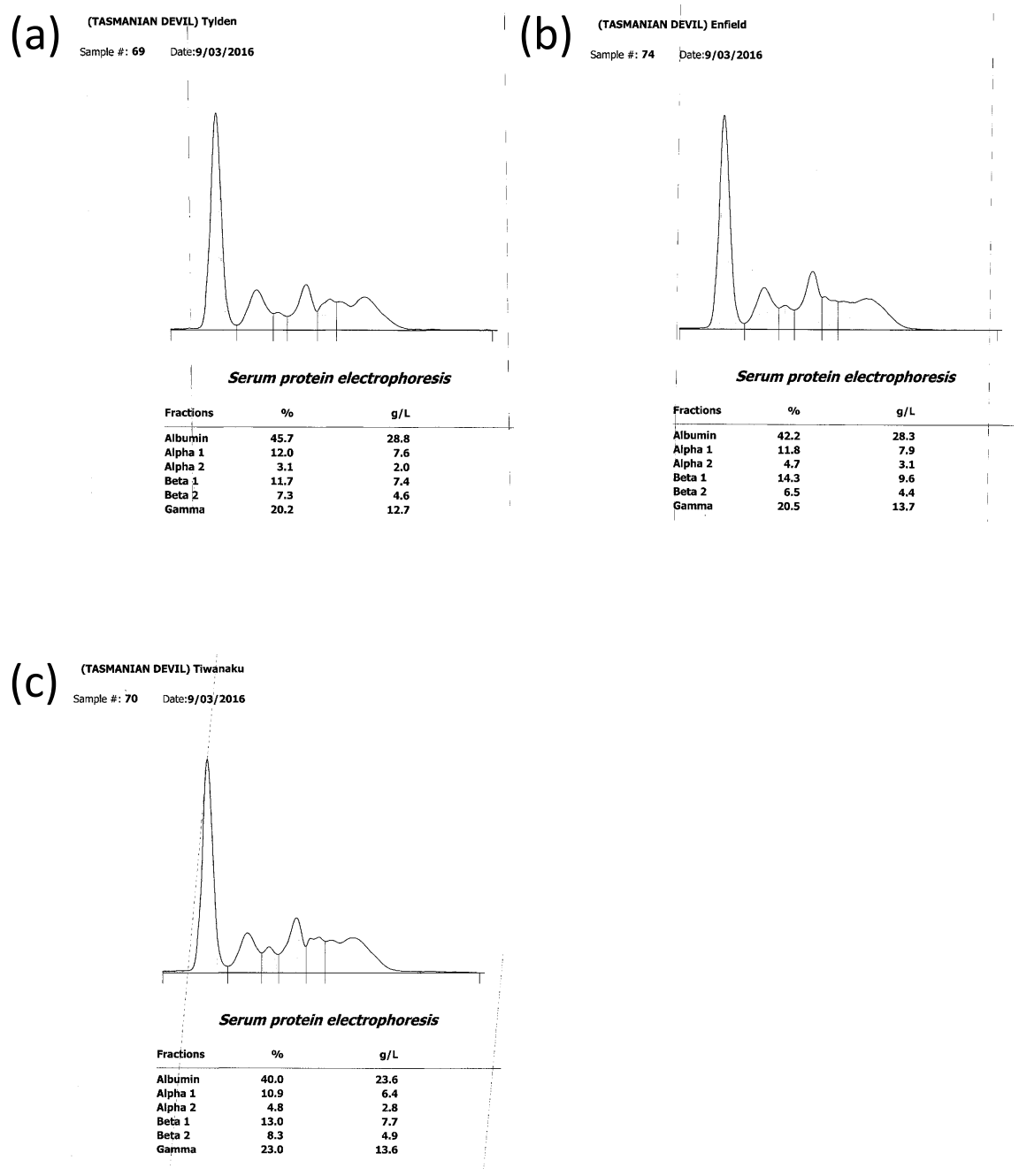
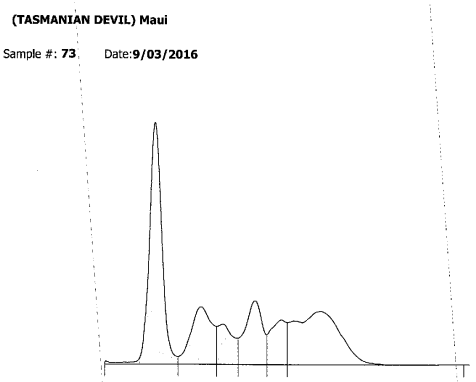


Figure 3. Electropherograms of DFTD+ wild male devils (a) 1 year old, (b) 2 years old, (c) 4 years old

(a)

(TASMANIAN DEVIL) Maui

Sample #: 73 Date:9/03/2016



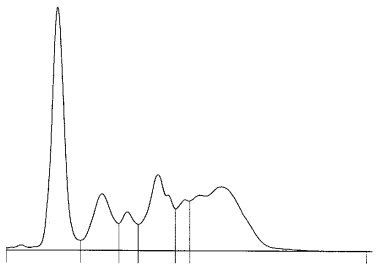
Serum protein electrophoresis

Fractions	%	g/L
Albumin	34.2	22.9
Alpha 1	13.4	9.0
Alpha 2	6.8	4.6
Beta 1	12.0	8.0
Beta 2	7.6	5.1
Gamma	26.0	17.4

(b)

(TASMANIAN DEVIL) Savuti

Sample #: 71 Date:9/03/2016



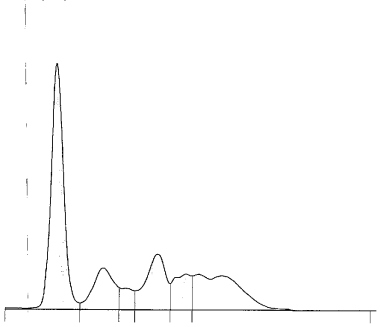
Serum protein electrophoresis

Fractions	%	g/L
Albumin	31.6	18.0
Alpha 1	11.4	6.5
Alpha 2	5.3	3.0
Beta 1	16.3	9.3
Beta 2	5.9	3.4
Gamma	29.5	16.8

(c)

(TASMANIAN DEVIL) Nungwi

Sample #: 72 Date:9/03/2016



Serum protein electrophoresis

Fractions	%	g/L
Albumin	43.8	28.5
Alpha 1	11.0	7.2
Alpha 2	3.5	2.3
Beta 1	13.9	9.0
Beta 2	7.8	5.1
Gamma	20.0	13.0

Figure 4. Electropherograms of DFTD+ wild female devils (a) 1 year old, (b) 2 years old, (c) 3 years old.

C reactive protein (CRP):

Although there were differences in CRP levels between the healthy and diseased devils, it is unlikely the actual values are significant. Active inflammation in domestic dogs is > 10 mg/L and the reliability of the test decreases with low values (< 5 mg/L) so the current method for CRP evaluation in dogs does not appear transferrable to devils.

Table 4. CRP ranges for healthy (DFTD-) and DFTD+ devils.

DFTD status	mg/L
Healthy (DFTD-)	0 to 0.58
DFTD+	0.79 to 4.91
Control (DFTD-)	0.86

where 0 value indicates below detection level or the test.

METHODS

Devil serum samples:

Sera samples from six DFTD+ and six healthy (DFTD-) devils from the West Pencil Pine devil population were used. Serum samples had been stored at -80 °C for up to two years, so a freshly collected serum sample from a captive healthy devil was also assessed. Samples were couriered frozen (on ice) from Hobart to Victoria, Australia.

Laboratory methods:

A commercial veterinary laboratory (Gribbles, Veterinary Pathology, Victoria, Australia) carried out the following analyses on each serum sample:

Albumin globulin ratio: A Siemens ADVIA 1800 analyser was used to measure the total protein and albumin content of each sample.
The total protein method is based on the method of Weichselbaum using biuret reagent (cupric sulfate in an alkaline solution).

The albumin method is based on the method of Doumas, Watson, and Biggs and uses bromocresol green solution (BCG) as a binding dye.
Globulin is not measured directly but calculated as the difference between TP and albumin

Protein electrophoresis: Electrophoresis was performed on the Sebia Hydrasys instrument using separation of proteins by their electrical charge on agarose gel and stained by amidoblack stain.

C reactive protein (CRP) method: A Randox Laboratories Ltd immunoturbidmetric assay for canine CRP quantification was performed on the serum samples.

Appendix 2

Information on wild Tasmanian devils from West Pencil Pine, north west Tasmania used in studies described in Chapters 3 and 4

Table 1. Wild devils from West Pencil Pine (WPP) used in Chapter 3.

Date/ DFTD status	WPP ID	Menzies ID	name	YOB	sex
Nov-13					
DFTD+	334	TD443	Kalahari	2011	F
	340	TD435	Ihaha	2011	F
	341	TD453	Limpopo	2011	F
	343	TD446	Savuti	2011	F
	369	TD449	Tylden	2011	M
	408	TD414	Alamo	2012	M
	410	TD418	Araucaria	2012	F
	416	TD437	Gaza	2012	F
	417	TD448	Fallujah	2012	M
DFTD-	349	TD441	Chiricoca	2010	F
	390	TD444	Karawinna	2012	F
	397	TD445	Pezgato	2012	M
	411	TD420	Acacia	2012	F
	414	TD440	Libano	2012	M
	422	TD442	Halhul	2012	F
	423	TD447	Albireh	2012	M
	424	TD451	Rafah	2011	M
Feb-14					
DFTD+	355	TD455	Zanzibar	2011	M
	360	TD438	Nungwi	2011	F
	382	TD415	Enfield	2012	M
	387	TD463	Kallista	2012	F
	420	TD452	Falestinya	2010	M
DFTD-	287	TD464	Nairobi	2009	F
	399	TD450	Chinchorro	2012	M
	409	TD417	Lenga	2012	F
	418	TD460	Persia	2012	F
	425	TD457	Il Bambino	2013	M
	428	TD456	Jujue	2013	F
	430	TD458	Chaco	2013	M
	433	TD459	Burruyacu	2013	F
	434	TD461	Catamarca	2013	M
	435	TD462	Formosa	2013	M

Date/ DFTD status	WPP ID	Menzies ID	name	YOB	sex
May-14					
DFTD+	283	TD421	Kisumu	2010	F
	381	TD433	Emerald	2012	F
	402	TD439	Selknam	2012	M
	403	TD473	Alacalufe	2012	M
	406	TD422	Aromo	2012	F
DFTD-	314	TD436	Trujillo	2009	M
	391	TD470	Kialla	2012	F
	404	TD474	Koru/ Chono	2013	M
	428	TD456	Jujue	2013	F
	437	TD472	Corrientes	2012	M
	438	TD475	Iguazu	2013	F
	448	TD478b	Pukunui	2013	F
	450	TD471	Waikaremoana	2012	F
	451	TD477	Rotarua	2013	F
Aug-14					
DFTD+	405	TD416	Tiwanaku	2012	M
	447	TD493	Maui	2013	F
	449	TD478	Kahurangi	2013	F
DFTD-	431	TD469	Mendoza	2013	M
	441	TD468	Aroha	2013	M
	442	TD496	Kia Ora	2102	M
	452	TD476	Taupo	2013	F
	455	TD491	Hobgoblin	2013	M
	456	TD492	Wheery	2013	M
	457	TD494	Hoppity Hare	2013	F
	458	TD495	Wandle	2013	F
	459	TD497	Old Speckled Hen	2013	F

Table 2. Wild devils from West Pencil Pine (WPP) used in Chapter 4.

WPP ID	Menzies ID	name	YOB	sex
69	/	Esquivel	2006	F
75	/	Gengibre	2006	F
138	/	Pomaire	2006	F
148	/	Ratusratus	2006	F
209	/	Oryx	2008	F
213	/	Mandrill	2008	F
214	/	Cobra	2007	F
219	/	Honeybadger	2008	F
220	/	Hornbill	2008	F
251	/	Monse	2009	F
253	/	Concepcion	2009	F
283	TD421	Kisumu	2010	F
287	TD464	Nairobi	2009	F
305	/	Evita	2010	F
314	TD436	Trujillo	2008	M
340	TD435	Ihaha	2011	F
341	TD453	Limpopo	2011	F
343	TD446	Savuti	2011	F
344	/	Churrete	2011	M
349	TD441	Chiricoca	2010	F
353	/	Matemwe	2011	F
355	TD455	Zanzibar	2011	M
358	/	Kendwa	2011	F
360	TD438	Nungwi	2011	F
381	TD433	Emerald	2012	F
382	TD415	Enfield	2012	M
387	TD463	Kallista	2012	F
399	TD450	Chinchorro	2012	M
400	TD419	Diaguita	2012	F
402	TD439	Selknam	2012	M
403	TD473	Alacalufe	2012	M
405	TD416	Tiwanaku	2012	M
406	TD422	Aromo	2012	F
408	TD414	Alamo	2012	M
409	TD417	Lenga	2012	F
410	TD418	Araucaria	2012	F
411	TD420	Acacia	2012	F
414	TD440	Libano	2012	M

WPP ID	Menzies ID	name	YOB	sex
416	TD437	Gaza	2012	F
417	TD448	Fallujah	2012	M
418	TD460	Persia	2012	F
420	TD452	Falestiniya	2010	M
422	TD442	Halhul	2012	F
423	TD447	Albireh	2012	M
424	TD451	Rafah	2011	M
429	/	Salta	2013	F
430	TD458	Chaco	2013	M
431	TD469	Mendoza	2013	M
433	TD459	Burruyacu	2013	F
434	TD461	Catamarca	2013	M
435	TD462	Formosa	2013	M
438	TD475	Iguazu	2013	F